STUDIES ON THE HISTOLOGICAL AND BIOCHEMICAL CHANGES DURING SPERMATOGENESIS IN *MUGIL CEPHALUS* LINNAEUS AND RELATED SPECIES

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D E C L A R A T I O N

I hereby declare that this thesis entitled "STUDIES ON HISTOLOGICAL AND BIOCHEMICAL CHANGES DURING SPERMATOGENESIS IN MUGIL CEPHALUS LINNAEUS AND RELATED SPECIES" has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

Cochin-682 031
February, 1987

Elizabeth Joseph

CERTIFICATE

This is to certify that the thesis entitled "STUDIES ON HISTOLOGICAL AND BIOCHEMICAL CHANGES DURING SPERMATOGENESIS IN MUGIL CEPHALUS LINNAEUS AND RELATED SPECIES" is the bonafied record of the work carried out by Kum. ELIZABETH JOSEPH under my guidance and supervision and that no part thereof has been presented for the award of any other degree.

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C O N T E N T S

P R E F A C E

In the endeavour of rational exploitation of fishery resources through the application of biological principles and intensive aquaculture of fishes through selective breeding, brood stock development, domestication and genetic improvement, studies on reproductive biology and physiology of fishes have attracted considerable attention. Among the different aspects of reproduction in fishes, gametogenesis forms an important and vital phase. During this phase, certain cells in the gonads transform through a series of morphological and cytological events into specialised cells or gametes namely, ova in the female and sperms in the male. The formation of the male gamete is known as spermatogenesis and that of the female, oogenesis. Spermatogenesis begins with the sexual differentiation of the fish and continues through the development and maturation of the testis. Although it occurs throughout the life of the fish, it is found to be active mainly during the breeding season.

Besides changes in the functional morphology of the testis, spermatogenesis also brings about considerable variation in the physiology and biochemical composition

of the fish. The salient features of spermatogenesis and the associated biochemical changes occurring during the process, in two of the important cultivable fishes namely, Mugil cephalus Linnaeus and Liza parsia (Hamilton - Buchanan) belonging to the family Mugilidae form the subject matter of this thesis.

The fishes belonging to the family Mugilidae, commonly known as 'mullets', are widely distributed in the coastal waters and estuaries of the tropical and subtropical zones of all seas. A few species occupy the warm temperate and cool temperate zones (Thomson, 1966). They are also known to ascend the fresh water regimes of the rivers. Historically, mullets were described in the records of the fifteenth and sixteenth centuries. Their fisheries appear to date back to the times of the ancient Greeks and Romans. At present, they contribute to fisheries of varying magnitude in several regions of the world's coastal waters especially in south-west Asia, India, Meditrranean countries, East European countries, Central and South America and the Pacific basin (Nash and Shehadeh, 1980). In 1983, the total world landings of Mugilidae were estimated at 2,10,261 metric tons constituting 0.27% of the total world production estimated at 764,70,600 metric tons (FAO, 1983).

Besides contributing to the capture fisheries, mullets form one of the most extensively cultured group of fishes. In fact, it is opined that the significance of mullet resources lies not so much in the existing capture fisheries, but in their potential as cultivable fishes for extensive and intensive fish farming. It is presently cultivated in about fifteen countries in the world and has great potential for augmenting fish production through aquaculture and technology transfer in many more countries.

Characteristics such as feeding low in the food chain, capacity to tolerate wide fluctuations in environmental conditions, fast rate of growth, limited breeding problems and their great demand as a delicious table fish, make mullets ideal for culture in different ecosystems of coastal sea water, estuaries, brackishwaters, and even in freshwaters.

In India, mullets are caught all along the coast in lagoons and creeks and in the adjacent estuaries and brackishwater lakes. The important fishing areas in the country are the estuaries of the rivers Ganga, Mahanadi, Godavari, Krishna and Cauvery and the brackishwater lakes of Chilka and Pulicat on the east coast; the estuaries of Narmada, Tapti, the Gulf of Kutch and the backwaters of Kerala on the west coast. In 1985 the estimated landings of mullets in the

country were 5,092 tonnes, forming 3.23% of the total marine fish production of India (CMFRI, personal communication).

Mullets form an important constituent in the catches of traditional brackishwater fish culture operations practiced in West Bengal, Kerala, Karnataka and Goa. They are cultivated in the low lying fields near estuaries and deltaic areas, as well as in paddy fields. Although, the production in these culture systems has been relatively low due to poor management, unsatisfactory water supply, unscientific stocking, lack of proper food and limited period of culture, recent efforts on mullet culture in specially prepared farms undertaken at Cochin has given encouraging results. The availability of vast areas of coastal waters suitable for culture and large number of fry and fingerlings in the inshore waters, estuaries and backwaters project immense prospects for large scale culture of mullets in the country.

Among the different species of mullets occurring in the world, M. cephalus is the most common, widely distributed species. It has gained considerable importance as a candidate species for aquaculture in several parts of the world. In India too, M. cephalus is the most widely distributed species occurring along both the coasts and is considered to be the foremost

species of finfishes having great prospects and potentials for aquaculture. L. parsia, the other species of mullet treated in this presentation is a medium-size fish, reported to reach a maximum size of 330 mm. It supports the local fisheries in Hooghly-Matlah estuary, Mahanadi estuary, Pulicat lake and in the south-west coast of the country.

Several studies on the biology and fisheries of mullets, particularly of M. cephalus are now available. Different aspects of breeding, larval rearing, seed production, field culture and ecophysiology have also been investigated. However, information on the spermatogenesis in M. cephalus as well as L. parsia is scanty. Since an understanding of the reproductive strategies is an essential pre-requisite for evolving successful breeding programmes through artificial fertilization and gametic preservation, investigations on spermatogenesis in these species were taken up and the results are presented in this thesis.

The thesis is presented in 9 chapters. Chapter 1 surveys the important literature relating to the taxonomical considerations and biology of mullets in general. Reviewing the works carried out on the reproductive physiology of mullets, the background and objective of the present study are given. After presenting the various methodologies followed in the study in

chapter 2, the morphological, anatomical and histological organisation and structure of the male reproductive system of M. cephalus and L. parsia are detailed in chapter 3. In chapter 4, the maturation process of the testis from the immature to mature and spent stages along with the salient characteristics of each stage are considered. Chapter 5 draws attention to the complex interrelationships between the reproductive effort and environmental factors in M. cephalus and L. parsia as revealed by the data collected from the Cochin estuarine area. The cellular changes occurring during spermatogenesis from the primordial germ cell stage upto the spermatozoa in both the species are traced and discussed in chapter 6. Chapter 7 deals with the biochemical changes occurring in certain somatic tissues and the testis with respect to maturation. The results of the histochemical characterisation of the testis at different stages of maturity are discussed in chapter 8 and finally in chapter 9, studies carried out on the cryopreservation of milt of both the species and the possibilities of using cryopreserved milt in the breeding programmes are presented and discussed.

The study reveals that M. cephalus has two distinct peaks of spawning between November and May. The prevailing environmental parameters do not seem to influence the spawning or maturation of these fishes in

a profound manner. Under natural conditions, the reproductive cycle seems to be timed by an endogenous rhythm. The testes of M. cephalus and L. parsia belong to the lobular type with unrestricted distribution of spermatogonia. The cellular details as revealed by light and electron microscopic studies show that spermatogenesis and spermiogenesis follow the same course in both the species, although the size of the individual cell types and the nucleo-cytoplasmic ratios vary. The spermatozoa of both the species lack acrosome. The formation of the flagellum, and its mode of attachment to the sperm head indicates that the mullet spermatozoa are not highly evolved. During maturation, protein, carbohydrate and lipid from the soma get translocated to the testis for the synthesis of gametes and reproductive hormones. An increase in the staining intensity of basic proteins is seen in the spermatids and sperms during the final stages of spermatogenesis. Lipids were mostly localised in the connective and interstitial tissues of the testis while carbohydrates were detected in traces in most of the cell types of the testis. All these as well as the observations made on cryopreservation of milt and their successful use in fertilizing the eggs of L. parsia, form the original contributions of the present investigation. The results of the studies thus add not only to the understanding

of the basic aspects of reproduction of these important cultivable fishes but also to the applied aspects relating to the development of artificial propagation techniques for intensive culture, inorder to meet the ever increasing demand for fish and fishery products.

A C K N O W L E D G M E N T

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CHAPTER I

INTRODUCTION

Contemporary species of fishes belonging to the family Mugilidae are assigned to fourteen genera with sixty four species (Thomson, 1981). Although, the taxonomical interest of mullets dates back to the time of Linnaeus in the 18th century, the identity and the taxonomic status of the different species have been a subject of contention. The wide geographical distribution exhibited by the mugilid species and the local variations in the morphological features have greatly contributed to this situation. The criteria employed for identity at the generic and species levels are also found to differ with different workers. Thus, Schultz in 1946 showing the anatomical uniformity of the group, relegated the previously recognised thirty seven genera of mugilids to thirteen genera on the basis of characteristics of mouth parts. Thomson (1954) working on the mugilids of Australia and adjacent seas distinguished thirteen genera and based the identity on the pattern of dentition and facial characteristics. The other morphological features used for the distinction of the species of Mugilidae are the otoliths (Morovic, 1953; Erman, 1960), adipose eyelid, lips, nostrils,

pyloric caeca (Hotta and Tung, 1966; Luther, 1975) and the vertebral column (Luther, 1975). Attempts were also made to distinguish the species on the physiological and biochemical basis. Herzberg and Pasteur (1975) distinguished five species of mugilid from the eastern Mediterranean on muscle proteins, while Gunter et al. (1961) and Senkevich and Kulikova (1970) observed differences in plasma protein and serum protein respectively, in M. cephalus and related grey mullets. Eye-lens proteins were also studied to identify the isolated mullet population in Hawaiian waters by Peterson and Shehadeh (1971). Thus, despite the several accounts, discussions and reviews (Schultz, 1946; Thomson, 1954, 1966, 1981; Trewavas and Ingham, 1972) available at present on the taxonomy of the family, the status of the different genera and species included in the group still remains confused. Nevertheless, several keys are now available for the identification of the adult species from different regions (Thomson, 1954; Bograd, 1955; Ebeling, 1957; Trewavas and Ingham, 1972 ; Ben-Tuvia, 1975; and FAO, 1971 and 1974).

Studying the geographic distribution of mugilid species which inhabit the tropics and subtropics with some species in warm temperate zones and a few penetrating the cold temperate waters, Thomson (1966) observed ten genera with fortynine species distributed in the

Indo-Pacific region, two genera and six species in the north-east Atlantic, three genera and nine species in the south-east Atlantic, two genera and seven species in the west Atlantic and three genera and five species in the east Pacific.

Mullets constitute a remarkable group of fishes in that they inhabit different ecosystems such as inshore sea, estuaries, brackish waters and fresh waters. The species typical of the fresh water habitat belong to the genera Trachystoma, Agonostomus, and Rhinomugil. M. cephalus is found to be the most tolerant among the mullets, the range of salinity tolerance of the species being from trace to 113 ‰ (Zenkevitch, 1963; Odum, 1970). The lower limit of salinity tolerance of L. ramada was found to be 5‰, of L. provensalis 10‰, of L. saliens 16‰ and L. aurata 24‰ (Brunelli, 1916). Although information on the temperature tolerance of mullets is scanty, it is reported that M. cephalus does not inhabit water below 16°C (Thomson, 1966). Several of the authors consider this species as diadromous.

A wealth of information is now available on the biology of grey mullets, particularly of M. cephalus which is the best studied species in the group, through the works of several investigators from different regions. The most important studies, to mention a few, are by Berg et al. (1949), Bograd (1961), Kristensen (1964),

Babayan (1965), Thomson (1966), Oren (1971), Ben-Yami (1974), Wallace (1975), Bruhlet (1975), De Silva and Wijeyaratne (1977), De Silva (1980), Yanez (1980), and Siva and De Silva (1981). In a comprehensive review Thomson (1966) summarised the existing knowledge on the habitat, morphological and anatomical features, reproduction, age and growth, population structure, behaviour, migration and fisheries of the group upto that time. This was followed by an excellent edition by Oren (1981) on the "Aquaculture of Grey Mullets" wherein aspects such as taxonomy, reproduction, age and growth, food and feeding, energy metabolism, artificial propogation, parasites and diseases and aquaculture methods of grey mullets were dealt with.

Sexuality and reproductive biology of grey mullets have been the subject matter of several investigations and have been reviewed by Thomson (1966) and recently by Brusle (1981a). Mullets are heterosexual fishes, but occasional abnormalities and hermaphroditic conditions have been recorded. The structure and development of the gonads of M. cephalus was described by Stenger as early as 1959. Thong (1969) studied the microscopial development of the gonads of M. auratus, M. chelo, and M. capito. The gonadal development of the latter two species was also investigated by Cassifour (1975). Recently ultrastructural studies on gonadial tissue of mullets were carried out by Van der Horst and Cross(1978)

on L. dumerili, Brusle and Brusle (1978a, 1978b) on L. auratus and Brusle (1980) on M. cephalus. Several studies have also been conducted on the oogenesis and vitellogenesis of M. cephalus (citing recent references: Kuo et al., 1974b; Kuo and Nash, 1975; Timoshek and Shilenkova, 1974) and of M. auratus, M. chelo and M. capito (Thong, 1969; Donato and Contini, 1974a, 1974b).

Majority of the mullets are known to prefer brackish water for growth and sea water for breeding. The seaward migration for spawning purposes have been recorded by Wimpenny and Faouzi (1935), Breder (1940), Broadhead (1953), Dekhnik (1953), Arnold and Thompson (1958), Jhingran (1958, 1959), Jhingran and Mishra (1962), Patnaik (1966) and Wallace (1975). However, Roughley (1916) believed that M. cephalus spawn in fresh water while Smith (1935), Breder (1940) and Jacob and Krishnamurthy (1948) opined that they spawn in the estuaries and tidal creeks. Kesteven (1953) reported the breeding of this species in the surf zone of Australian waters.

A perusal of literature for information on the pattern of sex ratio distribution, spawning areas and grounds, spawning frequency and seasons reveals that the observations of different workers are inconsistent and often controversial (Brusle, 1981a). In the marine region, M. cephalus is reported to breed in the littoral zone,

although Arnold and Thomson (1958) have shown that the species spawns in surface waters in the continental slope off the south-east of Mississippi delta where the depth is 750 fathoms. Breeding in the offshore grounds at varying depths is also recorded for M. curema and M. auratus. Most of the species are found to spawn in periods of low or declining temperature, salinity and photoperiod.

The fecundity in mullets is found to vary greatly and depends on the species, its size, region and period. In M. cephalus, the number of eggs produced is estimated to vary from one million to seven million. Mugilid eggs are pelagic/relatively small. The eggs and larvae of /and mullets are found abundantly in the coastal waters (Sanzo, 1930, 1936; Panikkar and Nair, 1945; Bal and Pradhan, 1946, 1947, 1951; Basu, 1946; Nair, 1946, 1952; Nair, 1957; Chacko and Ganapathi, 1949; Pillay and Shaw, 1949; Chacko, 1950; Rabanal, 1951; Pakrasi and Alikunhi, 1952; Sarojini, 1958; Kuthalingam, 1961; Sehgal, 1961; Basheeruddin and Nayar, 1961 and Thomson, 1966). As the young ones reach a size of 17 to 25 mm they enter the estuaries. Zismann (1981) has discussed the various characters of identification of fry and fingerlings of grey mullets.

The food and feeding aspects in grey mullets in the natural habitat and under artificial conditions have

been investigated by several workers and reviewed by Pillay (1953), Thomson (1963, 1966), Odum (1968a, 1968b, 1970), Hickling (1970), Zismann et al. (1975) and Brusle (1981b). In the juvenile stages, mullets feed on a wide variety of organisms of both plant and animal origin. While diatoms form the main component of the plant material ingested by the juveniles, planktonic and benthic organisms such as copepodes, ostracods, amphipods, isopods and zoea larvae constitute the main content of the zooplankton taken at this stage. The carnivorous habits of the mugilid larvae have been described by Odum (1970). In the adult stage the mullet is found to feed on all available food and their different habits of feeding have led to them being described as 'algal feeders' by Haitt (1944), 'iliophagus' by Pillay (1953), 'detritus feeders' by Rajan (1964), 'feeders on micro-and meio-benthos' by Hickling (1970), 'interface feeders' by Odum (1970), 'deposit feeders' by Fagade and Olaniyan (1973), and 'soft bottom feeders' by Blaber (1976). However, they can be generally considered as herbivorous feeding on algae and detritus, but also found to feed on zooplankton and zoobenthos. Thus feeding at the lowest trophic level, they play significant role in the flow of energy in the ecosystem.

The age and growth of mullets were studied mainly on the basis of interpretation of annuli observed in scales

and otoliths. The available information on this aspect reviewed by Thomson (1963) and Brusle (1981a) showed, the wide disparity and disagreement on the age and growth rates of different mullets. The studies of Anderson (1958) and Broadhead (1958) showed that M. cephalus attains a length of 160 mm standard length (140-170 mm length to caudal fork) at the end of one year. The age at first maturity of M. cephalus according to the most favoured view is two years for males and three years for females. The smallest size at maturity have been recorded in fishes from warm waters and the largest in fishes from the cold waters (Brusle 1981a).

Recently Paperna and Overstreet (1981) excellently reviewed the parasites and diseases of mullets and the public health aspect of 'mullets as toxicants to man'. Mullets, particularly M. cephalus are susceptible to bacterial (caused by Pasteurella-like bacterium, Streptococcus, Achromobacter spp) and fungal (Sparolegnia spp) diseases. Protozoan flagellates such as Amyloodinium ocellatum and Oodinium cyprintum, ciliates (Trichodina spp), sporozoans, microsporidians and myxosporidian parasites, copepods (Ergasilid spp, Somolochus spp, Caligoid copepods), Argulus spp, isopods, gnathids, monogeneans, digeneans, cestodes, nematodes, and leeches are found parasitic on mullets, and to adversely affect the different life activities, often causing mortalities especially in farming systems.

Mullets contribute to a capture fishery of considerable importance in about 24 countries in the world. The world catch of Mugilidae during the year 1983 was about 0.21 million tonnes of which M. cephalus contributed about 17.39% (FAO, 1983). The important countries where mullets are captured are Australia, Bulgaria, Burma, China, Egypt, Ethiopia, France, Hawaii, Hong Kong, India, Israel, Italy, Japan, Mauritius, Philippine, Portugal, South Africa, Spain, Taiwan, Thailand, Turkey, USA, USSR and Yugoslavia. The different methods of capture of grey mullets are discussed by Ben-Yami and Grofit (1981).

Historically farming of mullets have been in vogue as a traditional practice in the Mediterranean region, South-east Asia, Taiwan, Japan and Hawaii in the lagoons, creeks, swamps and ponds. Following a global awareness on the potentialities of mullets, particularly M. cephalus, among the cultivable marine and brackish water finfishes in developing the technology of aquaculture, a vast body of knowledge was accumulated on the cultivation of these fishes during the past three decades. Efforts were also mounted in different regions of the world not only to improve the traditional practice but also to introduce intensive systems of culture. Thus the traditional 'valli culture' methods of mullet in Italy were improved and advanced. The different mullet species introduced in

Egypt, USSR and Israel paved the way to establish the present day culture activities for these fishes in these countries. Intensive efforts are also made in several of the south-east and far-east countries, notably in Taiwan, Korea, Hong Kong, Indonesia and Malaysia, towards semi-intensive and/or intensive culture either in monoculture system or in polyculture along with compatible species.

The various studies carried out on the broodstock development, induced spawning, egg and larval rearing, larval nutrition, nursery management and culture techniques are reviewed recently by Nash and Shehadeh (1980) and Nash and Koningsberger (1981). Among the earlier accounts describing the culture techniques including the artificial propogation of mullets, particularly on M. cephalus, the works of Tang (1964), Kuo et al. (1973, 1974a), Nash et al. (1974) and Liao (1974) are the most outstanding ones.

Most of the Indian works on mullets pertain to the biology and fishery of the resources. One might refer to the earlier works on this aspect to the review by Sarojini (1951). The important later contributions were by Pillay (1954) and Sarojini (1958) on the mullets of West Bengal waters; by Luther (1963) on those of Mandapam region; by Patnaik (1966) and Rangaswamy (1973) on M. cephalus of Chilka and Pulikat lake respectively and by Sunny (1975) on the species of mullets from Kerala

waters. Luther (1968) and Kurian (1975) discussed the status and the fishery of the mullet resources in the country.

On the specific aspect of distribution of eggs, larvae and fry of mullets, the studies by Panikkar and Nair (1945), Nair (1946, 1952), Chacko and Ganapathi (1949), Chacko (1950) and Basheeruddin and Nayar (1961) from the Madras region and Bal and Pradhan (1946, 1947, 1951) from Bombay waters are noteworthy. Similarly food and feeding habits of mullets are discussed by Chacko (1949a, 1949b, 1949c), Mookerjee et al. (1946), Chidambaram and Kurian (1952), Pillay (1953) and Rajan (1964).

Another aspect of the biology of Indian mullets which has received considerable attention is on maturation and spawning. Studies on maturation process of gonads, particularly the ovary, has been carried out by Pillay (1948), Qasim and Qayyum (1961), Luther (1963), Patnaik (1966) and Rangaswamy (1975). As recorded in the other regions, the observations on spawning migration of the species in Indian waters is inconsistent. While Jhingran (1958, 1959), Jhingran and Mishra (1962) and Patnaik (1966) reported seaward migration for spawning, Kurian (1975) observed the movements in the reverse direction from the marine environment to the estuaries and brackish waters for spawning. Natural breeding of the species was studied

by Hora (1924), Jones (1946, 1950, and 1951), Basu (1946), Chacko (1950), Alikunhi (1957), Jacob and Krishnamurthy (1948) and Jones and Sujansigarai (1954). Hora (1938) and Pakrasi and Alikunhi (1952) reported that M. corsula could adapt completely to fresh water and breed in fresh water. Sebastian and Nair (1973, 1974) discussed the attempts made on artificial breeding of L. macrolepis at Cochin.

Investigations on the physiology of mullets of India are limited. Devanesan and Chacko (1943), Venkatraman (1944), Job and Chacko (1947) and Gosh (1967) reported on the acclimation of mullet from salt water to fresh water. Kuthalingam (1959) acclimated M. cephalus fry to different temperatures and concluded that they showed preferance for water at 29 to 32 °C. Kutty (1969) studied the oxygen consumption in L. macrolepis. Some aspects of energy metabolism of mullets, R. corsula, M. cephalus, and L. macrolepis, were also studied by Kutty and Mohamed (1975) and Kutty (1981).

The forgoing brief review of the literature on the investigations carried out on mullets in different regions of the world and from India shows that although a vast body of knowledge is now available on their reproductive biology, information on the development and maturation of the gonad at the cellular level and the intrinsic aspects of gametogenesis is largely lacking. This is particularly

so in respect of testicular constitution and spermatogenesis of grey mullets. Observing this lacunae in our knowledge and realising that an understanding of the maturation process of testes and spermatogenesis is an essential prerequisite for developing a successful artificial breeding programme, as well as in selective breeding strategies and its further application in genetic preservation of the resource, the present study on the spermatogenesis of M. cephalus and L. parsia along with the biochemical changes occuring in the fish during this process is taken up. The results of the experiments carried out on cryopreservation of the sperms of the two species are also reported.

Brief notes on the above two species are given below:

Mugil cephalus Linnaeus

This is the most widely distributed specimen of mullets in India and occurs in the sea, brackish water and freshwater regions. It supports a capture fishery of considerable importance at the Chilka lake, Pulicat lake, Mahanadi and Godavari estuaries, Gulf of Mannar and Palk Bay on the east coast and Kayamkulam and Vembanad lakes on the west coast. The females attain a maximum size of 900 mm and males 520 mm. The size at first maturity ranges from 200 mm to 430 mm in males and 240 mm to 570 mm in females. Males mature faster than females and are considered the dominant sex. The fry and fingerlings of

the species are found in abundance during the months of January - February and June - August in the west coast, November - February in the east coast and January-April in the Mahanadi estuary. M. cephalus forms an important candidate species of the traditional culture fishery practised in Kerala and Bengal.

Liza parsia (Hamilton - Buchanan)

This is a smaller variety of mullet with restricted distribution commonly found in the south-west coast of the Indian peninsula, below Bombay; the lower zones of Hooghly - Maltah and Mahanadi estuaries; and Pulicat, Vembanad and Kayamkulam lakes. The maximum size attained by the species is 330 mm. The size at first maturity is found to be 120 mm in males and 129 mm in females. Females are the dominant sex. The fry and fingerlings of the species are abundant in the months of January - April. The species contributes to the fishery mainly at the Hooghly - Maltah and Mahanadi estuary and Pulicat, Vembanad and Kayamkulam lakes. It also forms one of the species traditionally cultured in the brackish water ponds of Kerala and West Bengal.

CHAPTER II

MATERIALS AND METHODS

The specimens of *Mugil cephalus* and *Liza parsia* for the study were mostly collected from the Chinese dipnets located and operated at the Cochin bar mouth (Plates II and III Fig. 1). Some specimens were also collected from the brackish water fish culture ponds belonging to the Fisheries Department of the Government of Kerala at Malipuram in the Vypeen Island and from those belonging to the College of Fisheries of the Kerala Agriculture University at Panangad about 10 Kms from Cochin.

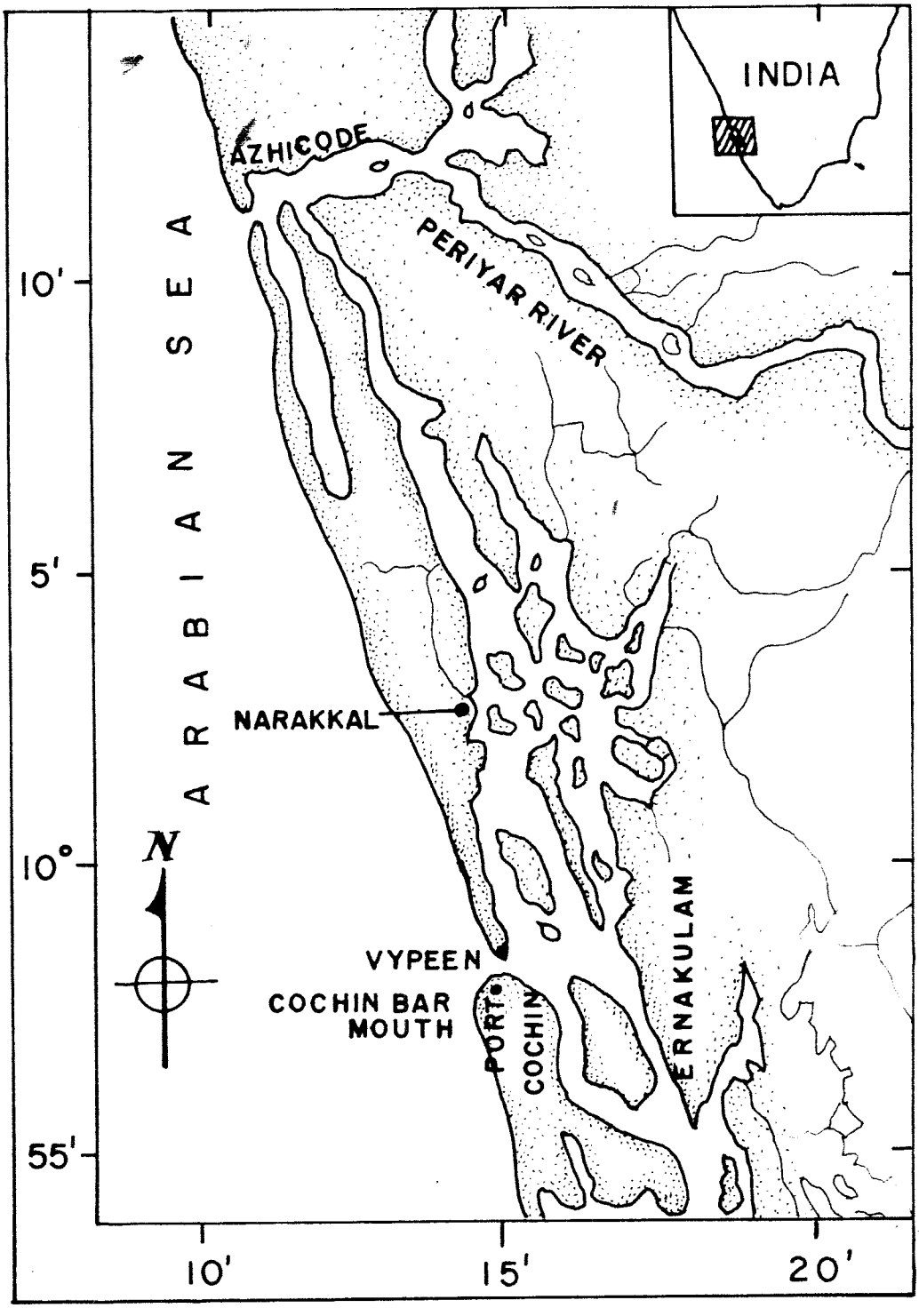
At the collection sites, the blood samples were taken from the live specimens by cardiac puncture using clean glass syringes pretreated with two percent tri-sodium citrate (anticoagulant) solution (Plate, III Fig.2). The samples thus collected were immediately transferred to labelled centrifuge tubes provided with suitable stoppers and kept in ice. The specimens were then preserved in ice contained in an ice box and brought to the laboratory for detailed studies.

During sampling, the surface water temperature at the collection site was recorded using an immersion

PLATE II

Map showing the collection sites around Cochin on the southwest coast of India from where the samples of *Mugil cephalus* and *Liza parsia* were collected for the study.

PLATE II


INDIA
AZHICODE
PERIYAR RIVER
ARABIAN SEA
10'
5'
10°
55'
NARAKKAL
N
VYPEEN
COCHIN BAR MOUTH
PORT COCHIN
ERNAKULAM
10'
15'
20'

thermometer (0 to 50° centigrade scale). Samples of surface water were also taken from the collection site, for estimating dissolved oxygen and salinity. The water samples for dissolved oxygen estimation was collected without agitation and fixed with Winkler's reagents as per the standard procedure. In the laboratory the salinity and the dissolved oxygen content were later determined by the titration method (Strickland and Parsons, 1968).

2.1. Morphology

In the laboratory, the specimens were sorted out into species and grouped according to size. After blotting out the water adhering to them, each fish was weighed and its total length (from tip of the snout to the tip of the caudal fin) and standard length (from tip of the snout to the end of the peduncle) were measured. Each fish was then dissected out and the gonads were examined. The colour, shape, length, breadth and weight of the testis and its volume in relation to the body cavity were recorded. Based on the volume occupied by the testes and their general macroscopical appearance, they were assigned to different maturity stages using a six stage maturity scale similar to the one adopted by Luther (1963) and Sunny (1975). The gonadosomatic index (GSI) was then calculated using the formula:

$$GSI = \frac{\text{Weight of the testes}}{\text{Weight of the fish}} \times 100$$

The condition factor (K) was calculated using the formula

$$K = \frac{W}{(L)^3} \times 100$$

Where W = weight of the fish in grams

L = length of the fish in cm

For studying the shape and size of the sperm in the milt of both the species, the following procedure was adopted. A small drop of fresh milt (about the size of a pin head) was placed on a clean dry glass slide along with a large drop of marine-fish-Ringer solution. A cover slip was carefully placed over it and the excess of solution blotted out with tissue paper. The slide thus prepared was observed under the phase contrast objective of an Olympus binocular (model VANOX-ABH-LB) microscope. The general structure of the sperm was recorded and measurements of the length and width of the sperm head and tail were made using a calibrated occular micrometer scale.

Smears of milt were prepared on glass slides, air dried and stained with 2 percent acetoorcein for three minutes. The stained slide was washed under running water and observed under Carl Zeiss binocular microscope at the magnifications of X 100 and X 400. Acetoorcein was found to stain the tails with good contrast.

2.2. Histology

For histological studies, portions of the anterior, middle and posterior regions of the testes dissected out from the freshly killed specimens were fixed in 10% neutral buffered formalin, Bouin's fixative or Zenker's fixative. After 24 hours of fixation, they were washed under running tap water and stored in 70 percent ethyl alcohol until further processing. Each tissue sample was given a code number and its details recorded. The stored tissues were later dehydrated following the standard procedure in graded alcohol series. The tissues were then cleared in chloroform or xylene, impregnated with and embedded in paraffin wax (BDH, 58–60°C melting point). The paraffin blocks were catalogued and stored in labelled polythene bags. Sections (longitudinal and transverse) were cut at 5–7/um thickness in a Fuji Optex (Japan) rotary microtome. Mayor's egg albumin (Gray, 1973) was used as the adhesive for fixing the paraffin ribbon with sections, on to the clean dry glass slides. The sections were deparaffinised, hydrated and stained with Heidenhain's iron alum haematoxylin as modified by Sprague (Clark, 1981), Harris' haematoxylin (Preece, 1972) and Lendrum's haematoxylin (Gray, 1973) with Eosin as the counter stain. Mallory's staining technique as modified by Lee Brown (Gray 1973) was also used in some cases. DPX was used as the mounting medium for all the slides. The sections were observed and photographed using a

Carl Zeiss Jena Ergaval binocular compound microscope, provided with mf camera attachment unit. The photomicrographs were taken using appropriate projection eye piece and 24 x 36 mm (100 ASA) negative film. The prints were taken on soft, glossy, single weight contrast paper and enlarged to the required size as per the instructions given in the Carl Zeiss Jena instruction manual (Carl Zeiss-No. 30-G 605g-2).

2.3. Transmission electron microscopy

The ultra structures of the various cell types of the testes of *M. cephalus* and *L. parsia* were studied by means of transmission electron microscopy at the Regional Centre of the Central Plantation Crops Research Institute at Kayamkulam, Kerala.

The testes tissue of various maturity stages, taken from live specimens of both the species were cut into small pieces and fixed immediately in 4 percent ice cold gluteraldehyde in Millonig's phosphate buffer (pH 7.2) at the site of collection. A few drops of milt collected from the live specimens of both the species were also fixed in the same manner. The samples thus fixed were transported to the laboratory in an ice box containing ice. In the laboratory, the fixed tissues were cut into smaller pieces and stored in fresh, cold fixative (4% gluteraldehyde in Millonig's phosphate buffer at pH 7.2) in a refrigerator at 4°C as suggested by Preece (1972).

Before processing, the gluteraldehyde fixed tissues were trimmed to smaller pieces (< 1 mm in thickness and 4 mm in length), given a fresh change of fixative and subjected to vacuum infiltration. They were then washed several times in Millonig's phosphate buffer (pH 7.2) and post-fixed in 2% osmium tetroxide solution prepared in Millonig's phosphate butter containing sucrose (pH 7.2) for two hours followed by two washes in double distilled water.

The samples for embedding in epon were dehydrated through a graded alcohol series (25%, 50%, 75%, 95% and 100%) at 4°C with fifteen minutes in each solution. They were then cleared in acetone (two changes of 30 minutes duration at room temperature), infiltrated with a graded series of epon in acetone (25%, 50% and 75%; one hour in the first two solutions and overnight in the last solution), given one change of two hours duration in 100% epon and finally embedded in epon. The embedded samples were subjected to a process of curing at 60°C for 36 hours.

When Spurr's embedding resin was used, the samples after rinsing with double distilled water were stained with 2% aqueous uranyl acetate for two hours at 4°C, washed twice with double distilled water, dehydrated in a graded series of acetone (20%, 50%, 70% and 95%) with

fifteen minutes duration in each grade. The tissues were then cleared by two changes of pure acetone (each of thirty minutes duration). The cleared tissues were infiltrated for two hours with a 2:1 mixture of acetone-Spurr's resin (Spurr, 1969) and then left overnight in 1:2 mixture of acetone-Spurr's resin. On the following day, each tissue was carefully transferred to the embedding capsules and fresh Spurr's embedding resin was poured in. These plastic capsules containing the tissue and resin, were allowed to remain at room temperature for one hour and subsequently incubated at 50°C for four hours and at 60°C for 48 to 72 hours in order to allow complete polymerisation of the resin.

The polymerised resin blocks, after removing from the embedding capsules, were trimmed with glass knife on LKB Ultratome III and a few semithin sections, 1 /um in thickness, were cut. These sections were stained by Methylene Blue - Azure II/Basic fuchsin staining technique (Humphry and Pitman, 1974), observed under a binocular compound microscope and the desired regions of the block for ultrathin sectioning were located. Block heads were further trimmed and ultrathin sections (600 to 700 Å) were cut by a freshly made glass knife attached to the ultratome. The ribbon of the ultrathin sections, was made to float in distilled water taken in a plastic boat fitted on to the glass knife. Using bent tipped forceps,

copper grids (300 mesh size) which were coated with 2% collodion solution in amyl acetate (Hayat, 1970), were carefully held below the ultrathin ribbon in the water and gently lifted so that the water seeped out and the sections adhered to the grids. After a few minutes of drying, the sections mounted on the grids, were stained with 2% uranylacetate in 50% ethyl alcohol for 15 minutes (Hayat, 1970). They were then rinsed thrice with glass distilled water, stained with 0.4% lead citrate in 0.1N NaOH for 5 to 10 minutes and treated with 0.02N NaOH for 5 seconds. These stained ultrathin sections mounted on the copper grids were screened under a Carl Zeiss transmission electron microscope EM 109 R and the required areas photographed on Agfaortho 25 negative film and printed on high contrast glossy paper with magnification, accurately controlled.

Samples of the milt suspensions of both the species preserved in the fixative (4% gluteraldehyde in Millonig's buffer) at 4°C, were directly taken on to copper grids (100 mesh size) coated with formvar and stained with 2% uranyl acetate stain for 30 seconds. They were subsequently screened under the Carl Zeiss transmission electron microscope and electron micrographs of entire sperms were taken.

2.4. Histochemistry

The distribution of the specific types of proteins, carbonydrates and lipids in the testes at various stages of development were studied using standard histochemical techniques (Mc Manus and Mowry, 1960; Pearse, 1968; and Subramoniam, 1982).

Neutral buffered formalin, Baker's solution and Carnoy's solution were used as fixatives. Fresh tissue squashes and cryocut sections of both fresh and fixed tissues were used for detecting lipids. For proteins and carbohydrates, the fixed tissues were processed, embedded and sectioned in the manner similar to that used for histology.

The presence of each reactive group was confirmed by simultaneously staining control sections subjected to blocking procedures for the specific groups. The histochemical tests and the corresponding blocking procedures are given in the relevant chapter.

As the testes of M. cephalus and L. parsia were composed of a large number of seminiferous lobules subdivided into cysts, the histochemical observations of the generative cells were essentially that of the collective reaction of all the cells in a single cyst. Since each cyst consists of only cells belonging to the same stage of spermatogenesis, the distinct reaction of each cell type could be recorded clearly.

2.5. Biochemistry

The amount of moisture, protein, carbohydrate, lipid and cholesterol in the tissues such as muscle, liver and gonad and in tne blood serum were determined at different stages of maturation by standard analytical methods, to understand the reproductive drain on these tissues and serum with respect to maturation.

Only specimens measuring above 25 cm (total length) in the case of M. cephalus and 9 cm in the case of L. parsia were taken for the analysis. All estimations were done on fresh tissues. In the case of all colorimetric estimations, the optical densities were read with an ECL senior spectrophotometer. All gravimetric estimations were made using a Wild Peinwage single pan electric balance.

Serum Analysis:

The samples of blood collected from the specimen at the collection site, were transported to the lab in an ice box containing ice. In the laboratory, these blood samples were centrifuged at 3000 r.p.m. for 10 minutes and the supernatant serum separated. When enough blood was not available from a single fish, blood samples of two or three fishes of similar size group and belonging to the same stage of maturity, were pooled before centrifuging.

0.2 ml of the serum was treated with 1.8 ml of 80% ethanol and centrifuged at 3000 r.p.m. for 5 minutes. The precipitate was used for estimating protein and the supernatent for estimating carbohydrate.

The precipitate was dissolved in 5 ml. of 1 N sodium hydroxide and one ml. of this solution was taken for estimating protein by the Folin - Ciocalteu phenol method (Lowry et al., 1951). After 20 minutes, the intensity of the colour developed was read at 700 nm. Bovine serum albumin was used as the standard.

Carbohydrate was estimated using the anthrone reagent (Roe, 1955). For this 0.5 ml of the supernatent was treated with 5 ml of ice cold anthrone reagent, mixed well and kept in a boiling water bath for 15 minutes. It was then cooled to room temperature in the dark and the intensity of the colour developed was read at 620 nm. Analar glucose was used as standard.

Lipids were estimated as per the method given by Folch et al. (1957). 0.4 ml. of the serum was extracted with chloroform - methanol mixture (2:1 v/v) and the extract thus obtained was mixed with a few drops of 0.9% NaCl and allowed to separate into two phases in a separating funnel. The lower phase containing chloroform and lipids was collected and lipid was estimated gravimetrically after evaporating the chloroform at 30°C in a vacuum desiccator.

Cholesterol was estimated by the ferric chloride acetic acid reagent method (Varley, 1962). 0.1 ml of serum was treated with 10 ml of ferric chloride-acetic acid reagent for 3 to 4 hours and centrifuged at 3000 rpm for 5 minutes. To 5 ml of the supernatent, 3 ml of concentrated sulphuric acid was added and the colour intensity developed was read at 560 nm. Chloroform (extra pure grade) was used as standard.

Tissue analysis: Moisture, protein, carbohydrate, lipid and cholesterol were estimated in the muscle, liver and gonad tissues of both the species of mullets at the different stages of maturity. The muscle tissue taken for estimation was white muscle from the dorsal part of the body, behind the dorsal fin. When sufficient tissue was not available from a single fish, tissues from different specimens belonging to the same stage of meturity were pooled together to make a sample for analysis. All estimations were made on fresh, wet tissues, since the methods adopted were found to be sensitive for estimating fresh, wet tissues rather than dried ones.

Moisture was estimated in approximately 100 mg of each tissue by gravimetric method after gradual dehydration at 60°C.

For protein and carbohydrate estimation, about 30-50 mg of each tissue was taken. Each tissue was homogenised with 5 ml of 5% trichloracetic acid, and

centrifuged. The precipitate was used for protein estimation while the supernatent was used for carbohydrate estimation.

The precipitate was dissolved in 5 ml of 1N NaOH. Protein was estimated in 1 ml of this solution by the Folin - Ciocalteu phenol method (Lowry et al., 1951). Bovine serum albumin was used as the standard. The intensity of the colour developed was read at 700 nm.

Carbohydrate was estimated in one ml of the supernatent from the above extraction, using freshly prepared anthrone reagent (Umbreit et al., 1964). The intensity of colour developed was read at 660 nm. In the case of liver samples, only 0.5 ml of supernatent was taken and made up to one ml with double distilled water, before adding anthrone reagent.

Approximately 40 mg of liver tissue, 60 mgs of muscle and 60 mg gonad tissues were used for lipid estimation. In each case, the tissue was homogenised with a mixture of chloroform - methanol in the ratio of 2:1 (v/v). The precipitate was filtered out and the extract was allowed to separate into the chloroform phase and the methanol phase in a separating funnel. The chloroform phase was collected and the solvent allowed to evaporate. The residual lipid was estimated gravimetrically (Folch et al., 1957).

For cholesterol estimation about 40-60 mg of tissue was homogenised with 5 ml of glacial acetic acid and centrifuged. To one ml of the supernatent, four ml of ferric chloride reagent was added and the mixture kept in ice. To the cooled mixture 4 ml of concentrated sulphuric acid was added and the colour developed was read at 540 nm.

Statistical analysis: The data obtained from biochemical analysis were subjected to statistical analysis. The analysis of variance was calculated for each biochemical parameter to test if there was any significant variation in the parameters (1) between the stages and (2) between the tissues in each stage.

The materials and methods employed for the cryopreservation study have been given in the relevant chapter.

CHAPTER III

ORGANISATION AND STRUCTURE OF THE MALE REPRODUCTIVE SYSTEM

Despite a good deal of investigations carried out on the reproductive biology and physiology of teleost fishes, there still exists considerable controversy regarding the structural organisation of the teleost testis, the terminologies employed and the identity and homologies of different cell types. Most of the works on these aspects have been reviewed by Hoar (1969), Dodd (1972), Lofts (1972), Lofts and Bern (1972), de Vlaming (1974), Guraya (1976), Callard et al. (1978), Hoar and Nagahama (1978), Grier et al. (1980), Billard et al. (1982) and Nagahama (1983).

The teleostean testis has been described as the 'lobular' type in some species and the 'tubular' type in others, by many authors. But no clear distinction between the two types was made until recently. In 1980, Grier et al., studied in detail the structural differences between the two types and described them as the 'unrestricted' type and the 'restricted' type. Billard et al. (1982) reintroduced the terms - 'lobular' type (unrestricted type) and 'tubular' type (restricted type).

Although Grier et al. (1980) included M. cephalus in the list of species examined during the study on testicular structure of fishes belonging to Salmoniformes, Perciformes, Cypriniformes and Antheriniformes, a detailed description on the structure and organisation of testes has not been given. It is, therefore, considered essential to study the structural organisation of the species under study, (M. cephalus and L. parsia) before taking up the detailed investigations on the process of spermatogenesis of these species.

OBSERVATIONS

Male Reproductive System of M. cephalus

Morphology

The male reproductive system of M. cephalus consists of a pair of elongated testes, vasa deferentia and a common sperm duct (Plate IV). The testes occupy about 75% of the body cavity in the mature condition. They are generally creamish white in colour. The two lobes of the testes are more or less of the same size (60 to 100 mm in length, 10 to 18 mm in width and 4 to 6 mm in thickness, in fishes ranging in size from 385 mm to 440 mm total length), though, occasionally one of the lobes of the testes is found to be slightly larger than

the other. In specimens weighing about 500 to 900 grams, the testes in the ripe condition weigh about 6 to 11 grams.

The testes appear to be laterally compressed, with the two lobes being almost uniform in width, except at the posterior ends, where they are found to taper slightly.

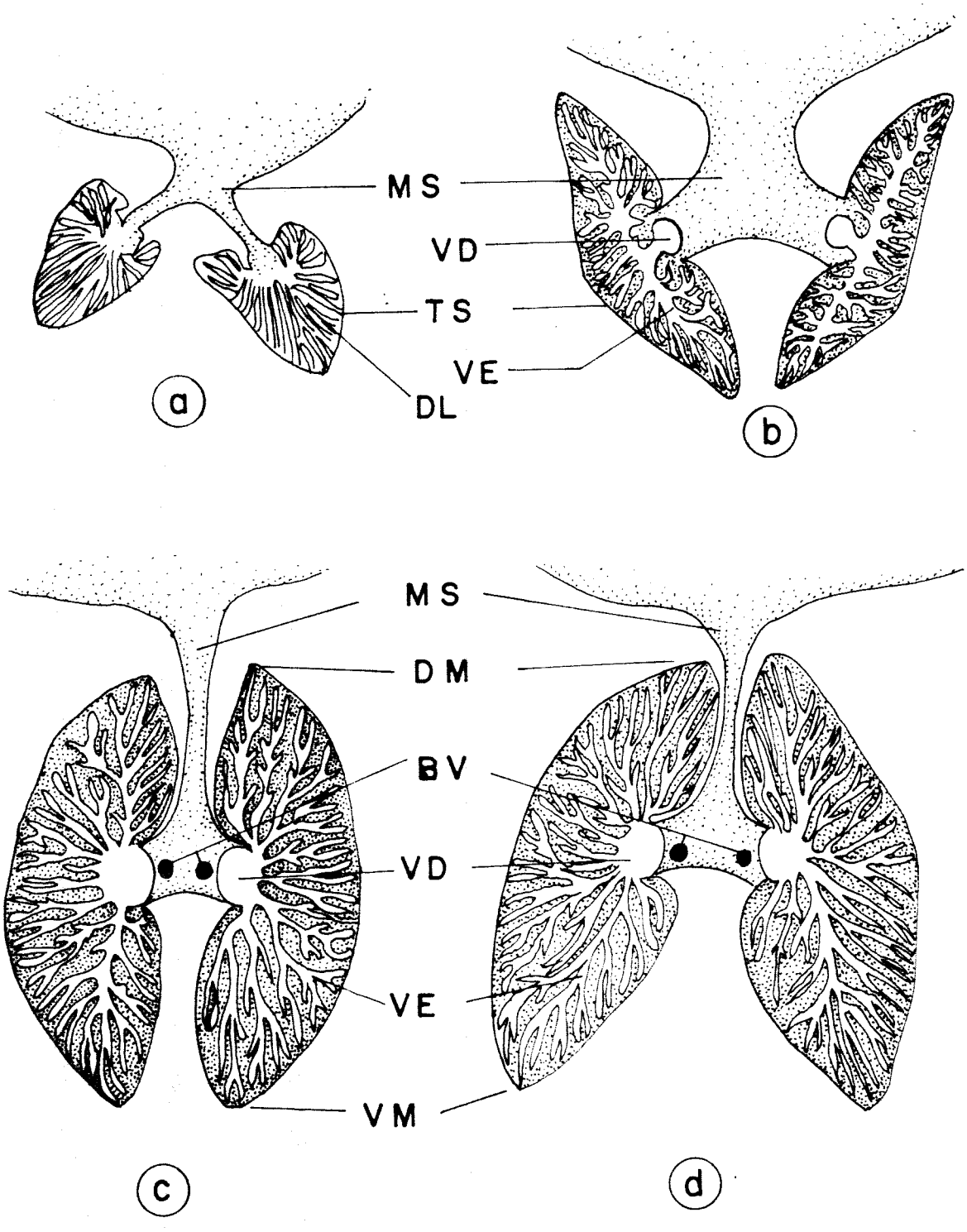
The testes are attached to the roof of the peritoneal cavity by means of connective tissue strands - mesorchium. In the immature condition (stage I) when the testes are thin and thread like, the attachment of the mesorchium appears to be from the dorsal side of the testes. As development proceeds, the testes lobes enlarge and get flattened. The attachment of the mesorchium then appears to be from the inner lateral side (Plate V). With further development of the testes, which are now laterally compressed, the mesorchium in between them becomes narrow and the testes get reoriented as shown in the figure (Plate V). The mesorchium also supports the genital blood vessels.

A vas deferens runs throughout the entire length of each of the testes along its inner lateral side. Posteriorly the two vasa deferentia are united to form a common sperm duct, which is covered by a connective tissue sheath formed by the mesorchium.

PLATE V

Figs. (a) to (d). Diagrammatic representation of the transverse sectional view of the testes, showing the gradual transition in shape and orientation of the testes with respect to the mesorchium. BV=Blood vessel; DL=Developing lobules; DM=Dorsal margin; MS=Mesorchium; TS=Testis; VD=Vas deferens; VE=Vas efferens; VM= Ventral margin.

PLATE V



No accessory reproductive organs are found in association with the male reproductive system in M. cephalus.

Internal Structure

In transverse section (Plate VI, Fig. 1) the mature testis appears to be kidney-shaped with the vas deferens situated in its concavity. From the vas deferens arise a number of primary and secondary ducts (vasa efferentia) that branch into the body of the testes. The vas deferens has a central lumen, with a diameter of about 1.32 mm, while the primary vas efferens has a lumen of 0.29 mm diameter. The diameter of the lumen of the ducts is found to vary and depend on the size of the testes and stage of maturity.

The terminal end of each vas efferens ends in a seminiferous lobule (Plate VI, Fig. 2). Each lobule has a central cavity that is continuous with the lumen of the vas efferens. All along the inner wall of the lobule are germinal cysts containing germ cells, namely, the primordial germ cells, the spermatogonia, the spermatocytes, the spermatids and the spermatozoa in various stages of development.

The cyst wall is made up of cytoplasmic extensions from the intra-lobular somatic cells (Sertoli cells) (Plate VII, Fig. 1). These cells are highly irregular, with

PLATE VI

Fig. 1. Transverse section of mature testis of *Mugil cephalus* showing the vas deferens (VD) and vasa efferentia (VE) filled with milt. BV= Blood vessel. Heidenhain's haematoxylin and eosin.

Fig. 2. Transverse section of mature testis of *Mugil cephalus* showing seminiferous lobules (SL) and vasa efferentia (VE)

their cytoplasm drawn into thin strands along the boundary of the germinal cysts. The cytoplasmic extensions from a single sertoli cell, may surround more than one germinal cyst and hence it is difficult to trace the entire cellular margin of a single cell.

The seminiferous lobules are seperated from one another by the basement membrane and inter-lobular somatic tissue. The basement membrane marks the outer boundary of the lobule. It is not always seen very distinctly because of the presence of a number of cells adjacent to it. Immediately outside the basement membrane lies a discontinuous row of spindle shaped cells (Boundary cells). A few connective tissue cells, blood vessels and Leydig cells are also seen in the inter-lobular space.

The Leydig cells frequently referred to as 'interstitial cells' by many authors, can be clearly seen only in the mature testis (Plate VII, Fig. 2). They are large polygonal cells usually seen in the interlobular somatic tissue at the junction of two or three seminiferous lobules.

The entire body of the testes is protected peripherily by a connective tissue capsule - tunica albuginea.

Male reproductive system of *L. parsia*

Morphology

The structure of the male reproductive system of *L. parsia* is similar to that of *M. cephalus*. In the mature fish, ranging in the size from 150 mm to 160 mm total length and weighing about 25 to 40 grams, the testes (Plate VIII) weigh about 100 to 300 mg and measure about 40 to 45 mm in length, 2.5 to 3.0 mm in width and 1 to 1.5 mm in thickness. It is creamish white in colour and twisted at various regions with the accumulation of milt. It is also turgid and oozes milt when slight pressure is applied to the abdomen. Two genital blood vessels run throughout the entire length of the testes in close association with the vas deferens. The vas deferens from both the testes unite posteriorly to form the common sperm duct, the terminal portion of which has a thick coat of connective tissue from the mesorchium.

Internal structure

The internal structure of the testes of *L. parsia* (Plate IX Figs. 1 and 2) resembles that of *M. cephalus*. The vas deferens sends out primary and secondary efferent ducts throughout the body of each testis. These ducts (vasa efferentia) lead to seminiferous lobules that end

PLATE VIII

Fig. 1. Male reproductive system of *Liza parsia*
CD= Common duct; TS= Testis.

PLATE IX

Fig. 1. Longitudinal section of the testis of *Liza parsia* showing vas deferens (VD); vas efferens (VE) and seminiferous lobules (SL). Harris' haematoxylin and eosin.

Fig. 2. Longitudinal section of the seminiferous lobules (SL) of *Liza parsia* (enlarged). SE= Seminiferous cyst; TA= Tunica albuginea. Harris' haematoxylin and eosin.

blindly near the peripheral margin of the testes, immediately within the tunica albuginea. The inner diameter of the vas deferens is about 0.23 mm and that of the primary vas efferens, about 0.064 mm. A large number of cysts lined by somatic cells (Sertoli cells) are distributed all along the inner margin and the terminal end of each seminiferous lobule. The Sertoli cells form the limiting boundary of each cyst. The cysts contain germ cells at different stages of development but within a cyst all the cells will be at the same stage of development. The interlobular somatic tissue consists of lobule boundary cells, Leydig cells, connective tissue and blood vessels. The cytoplasmic process of the Sertoli cells surround degenerating cellular components (residual bodies) and phagocitise them (Plate X).

DISCUSSION

Like most vertebrates, teleost fishes reproduce sexually, although hermaphroditism is reported occasionally. Gynogenesis is observed in some populations of *Carassius auratus* and also in *Poecilia formosa* (Hoar, 1965). In majority of the fishes, the gametes are released into the water and fertilisation is external. Internal fertilisation is reported only in Antheriniformes (e.g. *P. latipinna* Hurk *et al.*, 1974b, *Dermogenys pusillus* and *Horaichthys setnai* (Grier, 1981).

PLATE X

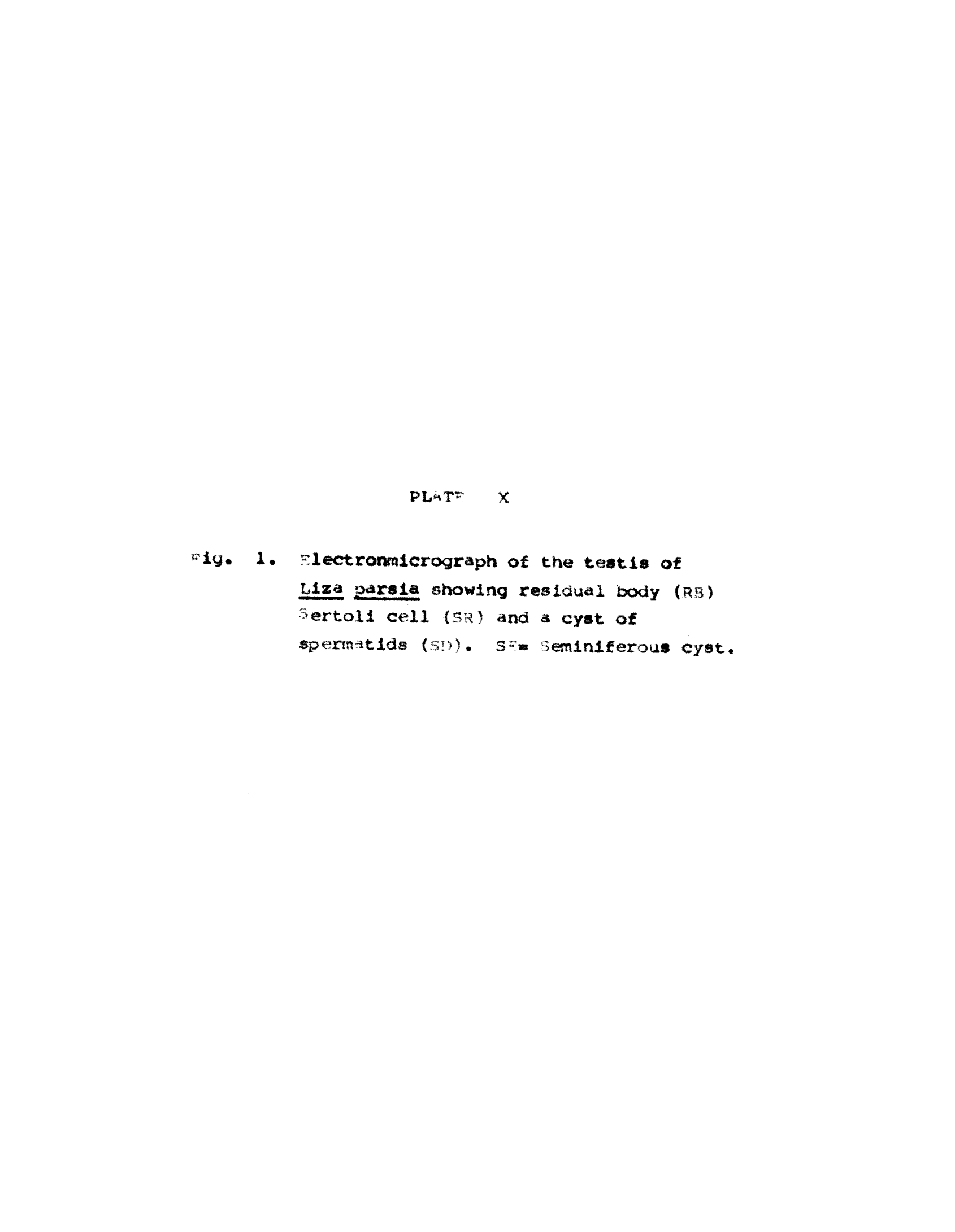
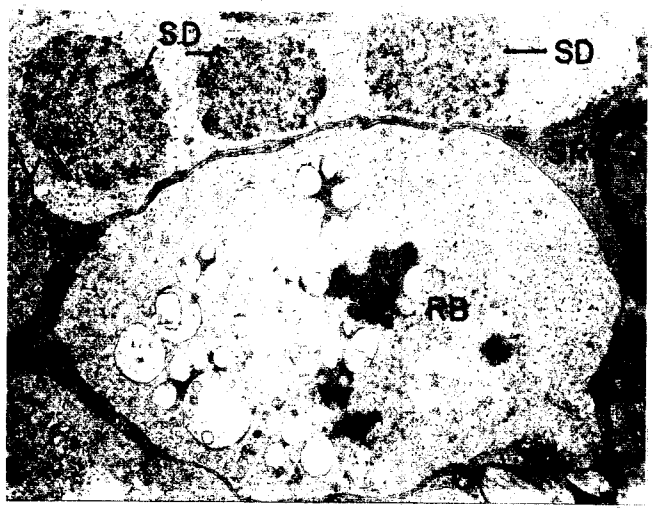


Fig. 1. Electronmicrograph of the testis of *Liza parsia* showing residual body (RB) Sertoli cell (SR) and a cyst of spermatids (SD). SF= Seminiferous cyst.

PLATE X



Unlike most vertebrate gonads that develop from two primordia (cortex and medulla), the teleost gonads (both ovary and testis) develop from cortex alone. This difference in the embryonic origin is considered as a reason for the occurrence of inter-sexuality among teleosts (Hoar, 1969).

In Mugilids, normally all members are heterosexual, but in some cases, inter-sexuality has been observed. Brusle (1981a) has given a brief review on this aspect. Orlandi (1902) observed an ovotestes in specimens of M. chelo collected from Ligurian area. Kestevan (1942) reported 'hermaphroditic roes' in the Australian mullet, M. dobula. In the case of M. cephalus, Johnson (1954) Stenger (1959) and Moe (1966) reported the presence of oocyte-like cells and ovotestes in the specimens caught from the Florida coast. Ovotestes was also described by Gandolfi et al. (1969) in M. saliens from Venice, by Thong (1969) in M. chelo and M. capito from Brittany, and by Brusle and Brusle (1975) in M. cephalus from Tunisia. In the present study, however, no sexual anomalies were observed among the specimens collected.

In mammals, the functional unit of the testes is considered as a seminiferous tubule. The corresponding unit in fishes has been described as a 'lobule' and a 'tubule' by different authors in different fishes. Unlike the mammalian seminiferous tubule, there is no permanent germinal epithelium in the teleost testes. Pointing out

this difference, Lofts and Marshall (1957) and Lofts and Bern (1972) considered it appropriate to call the teleostean male gonad as a 'lobular testes'. Though de Vlaming (1974) and Guraya (1976) did not consider any difference between 'lobular' and 'tubular' testes, Billard (1969), Pandey (1969) and Hurk (1973) identified a 'tubular' testis in P. reticulata, which was different from the 'lobular' testes found in other fishes. However, till 1980, there were no established criteria to distinguish between the two types of testes and the nomenclature used gave rise to a lot of confusion. As a result, the 'tubular' testes described by (Hoar (1969) in Fundulus, showed close resemblence to the 'lobular' testis described by Turner (1919) in Perca flavescens. In Salmo gairdneri (Salmoniformes), the testis was described as 'lobular' by Robertson (1958) and Oota and Yamamoto (1966) while Hurk et al. (1978a) considered it as 'tubular'. In F. heteroditus, Mathews (1938) and Lofts et al. (1966) described a lobular testis which was later described as tubular by Hoar (1969). Further, the terms 'lobule' and 'tubule' are used interchangeably without any distinction by some authors (Henderson, 1962; Sanwal and Khanna, 1972; Shrestha and Khanna, 1976, 1978; Dalela et al., 1976, 1977; Leatherland and Sonstegard, 1978).

In 1980, Grier et al. reexamined the testicular structure in three fishes belonging to Salmoniformes,

four to Cypriniformes, twelve to Perciformes and thirty one to Antheriniformes and reviewed the terminologies used for its description. The distribution of spermatogonia in the testes was taken as the basis for differentiating two types of teleostean testes - the 'restricted' type, in which the spermatogonia are restricted to the distal end of the tubule; and the 'unrestricted' type where spermatogonia are distributed all along the entire length of the tubule. The former type is found almost exclusively in Antheriniform fishes while the latter is commonly found in the Salmoniformes, Perciformes and Cypriniformes. Billard et al. (1982) and Billard (1982) taking into consideration the pattern of spermatogenesis, spermatology and reproductive physiology as the basis, distinguished a 'lobular' type testes (similar to the unrestricted type) and a 'tubular' type testes (similar to the restricted type). The term 'lobule' was preferred, since the diameter of the tube was variable and histologically it appeared like a lobe. The 'tubular' type was so called because of the regular form of the tubules, without any lumen within them.

In the present study, the structure of the testes in both M. cephalus and L. parsia were found to be of the lobular type following the description by Billard et al. (1982). The presence of germinal cysts all along the inner lobular wall indicates that the testes is of the 'unrestricted' type, as described by Grier et al. (1980) in the case of the fishes of the group, Perciformes. In a mature oozing testes of M. cephalus, the density of the germinal cysts along the

wall of the lobule is much less as compared to the number of cysts present at the terminal end of the lobule. Whereas in the case of L. parsia, the germinal cysts are more or less evenly distributed all along the wall of the tubule and its terminal end.

Considerable controversy also exists concerning identity and homology of different cells particularly Leydig and Sertoli cells in the testes of the teleost fish. In mammals, the steroid secreting activity of the gonad is carried out by the Leydig cells (situated interstitially) and the nutritive activity of the developing sperms is carried out by the Sertoli cells. The presence of cholesterol and lipid in the cytoplasm of the somatic cyst cells of teleost testis misled some workers (Marshall and Lofts, 1956; O'Halloran and Idler, 1970; Upadhyay and Guraya, 1971) to describe these cells as homologues of the mammalian Leydig cells and to refer them as 'lobule boundary cells'. Later workers (Stanley et al., 1965; Nicholls and Graham, 1972; Grier, 1976; Nagahama et al., 1978 and Hoar and Nagahama, 1978) however, considered these cells to be homologous with the Sertoli cells of mammals, based on their structural organisation and function. Grier et al. (1980) and Grier (1981) have been able to identify interstitial cells with secretary activity, that are similar to the true mammalian Leydig cells, in addition to the lipophilic Sertoli cells, in Esox lucius,

E. niger, Oncorhynchus kisutch, Notopis hypseloterus and Fundulus grandis. It is now clear that teleosts have both Sertoli cells and Leydig cells similar to those in mammals. The Sertoli cells may be either secretory or non-secretory in function. Billard et al. (1982), however, consider it more suitable to describe the cyst cells as 'intra-lobular somatic cells' rather than Sertoli cells.

In M. cephalus, Grier (1981) reports that"Leydig cells are difficult to find in the interstitium of fish undergoing recrudescence, but are reasonably common within the interstitium of fish at the nadir of the reproductive cycle". He has also been able to observe lipid droplets in the cytoplasm of the Sertoli cells prior to gonadal recrudescence but these were found to disappear once spermatogenesis commenced. In the present study, Sertoli cells and Leydig cells were observed in both M. cephalus and L. parsia. The cytoplasmic extension from the Sertoli cells were found to delimit the germinal cysts, within the seminiferous lobule. The cytoplasm, that was seperated from the developing spermatids and sperms were found in the form of 'residual bodies' (with a number of vesicles) being phagocitised by the Sertoli cells (Plate X). This phenomenon corresponds to that described by Grier et al. (1980) in F. grandis and F. seminolis.

In the Antheriniform fishes belonging to Poecilidae and Goodidae, which exhibit internal fertilisation, the

Sertoli cells play an important role in the formation of the spermatozeugmata and spermatophores e.g. H. setnai (Grier, 1981, 1984). Such a role of Sertoli cells is not observed in M. cephalus and L. parsia. In these fishes, the sperms are released freely into the lumen of the vas deferens and from there to the external medium, and not converted into specialised sperm packets (spermatozeugmata or spermatophore).

Unlike Sertoli cells that are found within the seminiferous lobule, the Leydig cells are found in the inter lobular tissue. They are usually found in few numbers at the junction of two or three lobules. Histochemical tests show that these cells are lipid positive. Apart from Leydig cells, the inter-lobular somatic tissue is made up of boundary cells lining the outer side of the seminiferous lobule in a discontinuous manner, fibroblasts and blood vessels. In most of the sections observed, the inter-lobular somatic tissue was so closely packed that it was difficult to differentiate each cell type. At the periphery of the testes, the interlobular somatic tissue becomes continuous with the connective tissue capsule - tunica albuginea.

The development and comparative anatomy of gonoducts in fishes have been reviewed by Hoar (1969) and Nagahama (1983). In teleosts, unlike in elasmobranchs and cytoclostomes, there is no connection between the gonoducts and the

mesonephros. The gonoducts are formed independently by somatic cells derived from the coelomic wall. Grier et al. (1980) while discussing the different arrangements of the testicular tubules and mainducts, mentioned the presence of a main spermatic duct in the dorsal surface of the testes in M. cephalus. The present study clearly revealed that, in M. cephalus, though the main sperm duct (vas deferens) is present in the dorsal position initially, as development proceeds, the testes increases in width and reorients itself in such a way that the mesorchium and the vas deferens now seem to be attached to the inner lateral side of the testis (Plate V). This condition is also observed in L. parsia. The main sperm duct (vas deferens) lying laterally, gives rise to primary vasa efferentia that extend dorsoventrally giving away smaller branches (secondary vasa efferentia) throughout the body of the testis.

The efferent duct system in M. cephalus and L. parsia (unrestricted type) is more elaborate than that found in the Antheriniform fishes (restricted type), which is more advanced than the type found in the elasmobranchs. In M. cephalus and L. parsia as in the case of other perciform fishes, the lumen of the efferent duct is continuous with the lumen of the seminiferous lobule, whereas, in the Antheriniform fishes, the efferent ducts do not penetrate deep into the testicular tissue, but end in solid seminiferous

tubules that have no lumen. However, in both the types (unrestricted and restricted) the germinal cysts are associated with the lobule or tubule, which is connected to the efferent ducts, unlike in elasmobrachs, where the seminiferous ampullae are present independently in the stroma of the testis and make contact with the efferent duct system only when the sperms are to be released from the ampullae.

In summing up, the testes of M. cephalus and L. parsia can be described as a lobular type with an unrestricted distribution of spermatogonia, an elaborate efferent duct system and secretory Sertoli and Leydig cells.

CHAPTER IV

MATURATION PROCESS AND MATURITY STAGES

In the study of the biology of fish, maturation process of gonads forms an important aspect as it leads to the understanding of the reproductive characteristics and breeding behaviour of the fish. In the earlier works only gross assessments of the maturity stages had been made based on the aspects such as growth of gonads, their relative size to the body cavity, oocyte diameter and colour of eggs. With advances made in the histological and cytological methods, the process of oogenesis and spermatogenesis have been studied in greater detail in recent years. Among the available literature on this aspect in the teleost fishes, special mention must be made of the works of the following authors : Dodds (1910), Turner (1919), Hickling (1935), Mathews (1938), Jones (1940), James (1946), Gosh and Kar (1952), Satyanesan (1960), Stanley et al. (1965), Stanley (1969), and Billard (1970c).

The maturation and spawning in grey mullets have been investigated by Breder (1940), Bromhall (1954), Thomson (1957), Luther (1963), Abraham et al. (1966, 1968), Thong (1969) and Hickling (1970). However, most of these works, generally deal with the development of ova

and the description of maturity stages in females. Luther (1963) studying the biology of grey mullets, described six maturity stages in both sexes of L. macrolepis mainly based on the size of the gonad with respect to the body cavity.

A perusal of the literature reveals that studies on maturation process of testes in mullets are restricted to the early light microscopic studies made by Stenger (1959) and a few papers published by Brusle and Brusle (1977a, 1977b, 1978a, 1978b) and Brusle (1980, 1981c). Although, M. cephalus is one of the important candidate species cultivated in different regions of the world, knowledge on the different aspects of gametogenesis of this species is scanty. This is especially so in the case of males. Since knowledge of these aspects forms an essential prerequisite for the effective management of brood stock, an attempt is made here to study the germ cell differentiation and maturation of the testes through different stages.

MATURATION OF TESTES

External morphology and maturity stages

Mugil cephalus

As in several teleost fishes, juvenile M. cephalus does not exhibit internal differentiation of sex. It

becomes discernible as the fish attains the size of about 20 cm. At this size, the males can be distinguished from the females by the presence of thin thread like, colourless strands of gonadal tissue, occupying the lower half of the body cavity. In the females, the ovary makes its appearance as pinkish, translucent ribbon-like tissue occupying one fourth of the posterior part of the body cavity.

In males, the process of spermatogenesis gives rise to simultaneous changes in the external shape, size and texture of the testes. Based on the external changes of the testes observed by macroscopic examination, the following six arbitrary stages of maturity have been identified.

Stage I (Immature) : The testes appear as two thin thread-like structures, united at the posterior end. They are semitransparent, colourless and measure about 5 to 7 cm in length and 1 mm in width. They occupy about half of the body cavity length-wise, from the posterior end (Plate XI, Fig. 1).

Stage II (Maturing I) : Each of the testes lobes at this stage gets flattened, appears opaque, with a smooth surface. On the inner lateral side of the surface of the testis, a furrow becomes

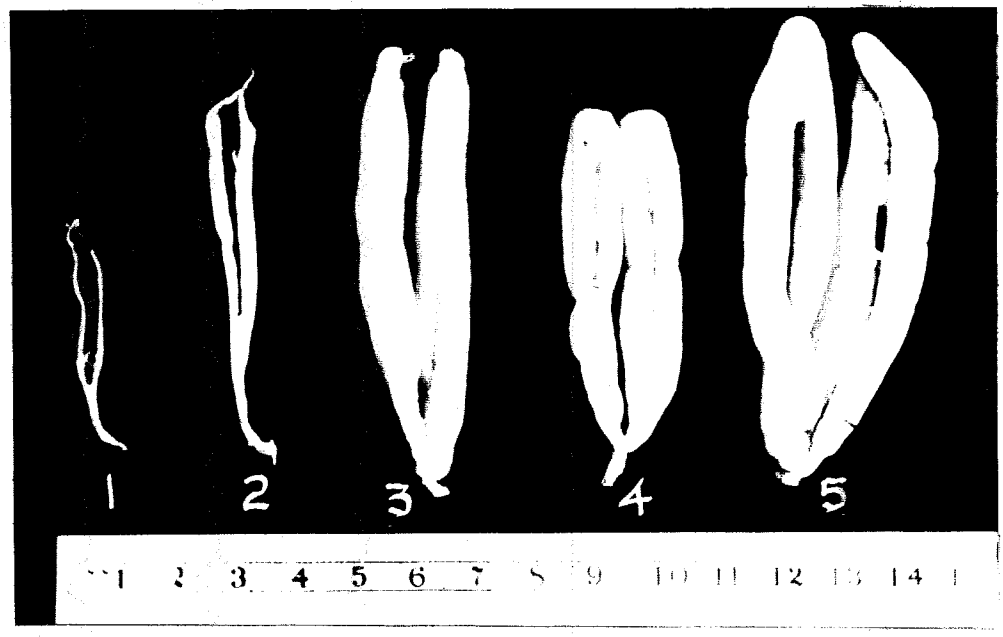
PLATE XI

Different maturity stages of the testes in *Mugil cephalus*.

1. Immature 2. Maturing I 3. Maturing II

4. Mature 5. Oozing

PLATE XI



discerinible at this stage due to the appearance of the vas deferens. The two lobes are unequal in length with a difference of about 0.3 cm between them. They measure about 5.5 to 6 cm in length and 2 to 3 mm in width. The entire gonad now occupies about two thirds of the body cavity of the fish (Plate XI, Fig. 2).

Stage III (Maturing II) : This stage is characterised by a pair of white, turgid and opaque testes with smooth surface. Each testis has a distinct vas deferens running throughout its entire length. The length of the testis in this stage varies from 6.5 cm to 8 cm and the width from 0.5 to 0.9 cm. They occupy about two thirds of the body cavity longitudinally (Plate XI, Fig. 3).

Stage IV (Mature) : Creamish white colouration and turgid nature of the testes with deep seated vasa defferentia mark this stage. The testes occupy about three fourths of the body cavity. The surface of the testes is smooth with a few twists here and there. In transverse section, each testis appears to be kidney shaped. The size of the testes almost is similar to that of the previous stage. But the width ranges from 1 to 1.5 cm. A small amount of milt oozes when pressure is applied to the abdomen (Plate XI,Fig. 4)

Stage V (Oozing) : The size, shape and colour of the testes is similar to the previous stage. But here the testes appear more turgid and the milt oozes out freely with slight application of pressure to the abdomen. Copious amount of milt oozes out from the cut ends of the testes (Plate XI, Fig. 5).

Stage VI (Spent) : The testes in this stage, appear semitransparent and flaccid with the surface thrown into folds. Only a little quantity of milt oozes out on application of considerable amount of pressure to the abdomen. Lengthwise, they occupy about two thirds of the body cavity. Each testis lobe measures about 6 cm in length and 0.6 cm in width.

Liza parsia

The gross external morphological features of the testes at the different stages of maturity in L. parsia are similar to that of M. cephalus. In L. parsia the clear distinction between male and female could be made only in fishes above 10 cm in length. The six stages of maturity distinguished on the basis of macroscopical examination are dipicted in (Plate XII).

Stage I (Immature) : The testes is bilobed, extremely thin, thread-like, transparent and occupies about

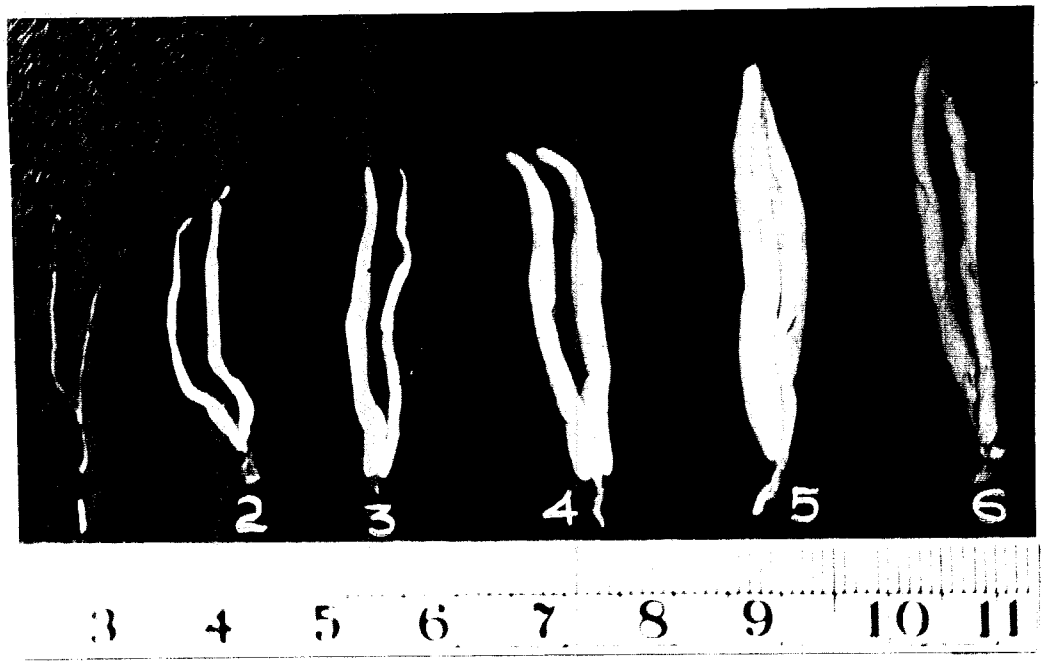
PLATE XII

Different maturity stages of the testes in *Liza parsia*.

1. Immature. 2. Maturing I. 3. Maturing II.

4. Mature. 5. Oozing. 6. Spent.

PLATE XII



fifty percent of the body cavity. Each lobe measures about 2.5 cm in length and about 0.5 mm in width. The two lobes are united posteriorly at the region of the urinogenital aperture.

Stage II (Maturing I): The testes at this stage is opaque, creamish-white, flattened and tape-like occupying two thirds of the body cavity. It measures about 3 to 3.5 cm in length. Each lobe has a width of 1 mm.

Stage III (Maturing II) : With the progress of maturation the testes appear white with distinct furrows of vasa defferentia. Each testis measures about 3.5 to 4.5 cm in length and 1.5 mm in width.

Stage IV (Mature) : The testes are turgid, slightly twisted, creamish-white, occupying about three fourths of the body cavity. Oozes very little milt on applying pressure to the abdomen. Length ranges from 4 to 4.5 cm and the width from 2 to 2.5 mm.

Stage V (Oozing) : Similar in appearance to the above stage. Oozes considerable amount of milt on applying slight pressure. Measures about 4 to 5 cm in length and 3 to 4 mm in width. Nearly fills the body cavity.

Stage VI (Spent) : Non-turgid, partly opaque and partly semi-transparent, with uneven surface. Measures about 3.5 to 4 cm in length and 1.5 to 2 mm in width. Occupies two thirds the body cavity.

Histological features

It is well known that the differentiation of primordial germ cells into gametes through the various developmental stages is an orderly process and follows a distinct pattern. To understand this pattern of maturation and sequence of development, histological studies of the testes of M. cephalus and L. parsia through different developmental stages were made. Further such studies would also help to clarify uncertainities in the fixation of maturity stages by external morphological features. The various histological characters of the testes in different maturity stages observed in the present study are described below.

M. cephalus

Stage I (Immature) : In the longitudinal section, the immature testes is found to be made up of connective tissue stroma in which isolated nests of large irregular primordial germ cells are distributed. The cells are all compactly packed witnin the nests, leaving no lumen (Plate XIII, Fig. 1).

PLATE XIII

Fig. 1. Longitudinal section of the immature testis of Mugil cephalus showing primordial germ cells (PG) and stroma of connective tissue (CT). Heidenhain's haematoxylin and eosin.

Fig. 2. Transverse section of stage II testis of Mugil cephalus showing initial stages in the formation of seminiferous lobules (SL). Harris' haematoxylin and eosin.

Fig. 3. Transverse section of stage II testis of Mugil cephalus (enlarged) showing early stages of cyst formation with primordial germ cells (PG), spermatogonia (SG) and somatic cells (CS). Harris' haematoxylin and eosin.

Fig. 4. Semithin transverse section of stage III testis of Mugil cephalus showing seminiferous lobules (SL) containing cysts of spermatocytes (SC), spermatids (SD) and isolated spermatogonia (SG). SE= Seminiferous cyst; IT= Interlobular somatic tissue. Methylene blue - Azure II/ Basic fuchsin.

Fig. 5. Semithin transverse section of stage IV testis of Mugil cephalus showing spermatogonia (SG), spermatocytes (SC), spermatids (SD) and spermatozoa (SZ). Methylene blue - Azure II/ Basic fuchsin.

Fig. 6. Transverse section of the stage IV testis of Mugil cephalus showing parachute like arrangement of sperms with the sperm heads (SH) to the periphery and sperm tails (ST) to the centre. LU = Lumen of the seminiferous lobule. Heidenhain's haematoxylin and eosin.

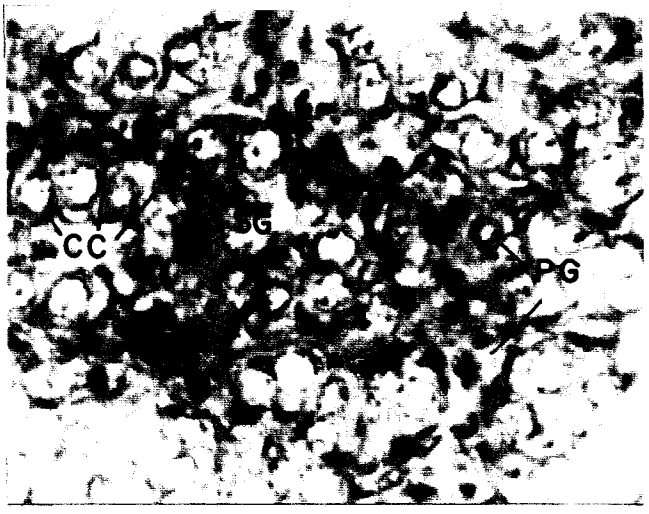
PLATE XIII

100 µm

1

70 µm

2

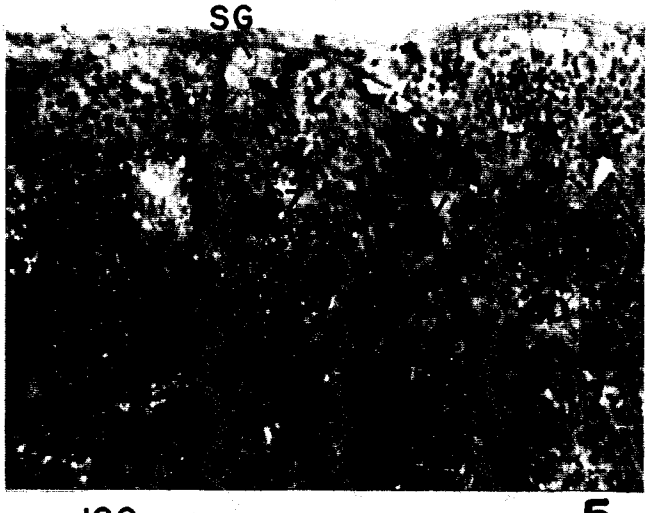


20 µm

3

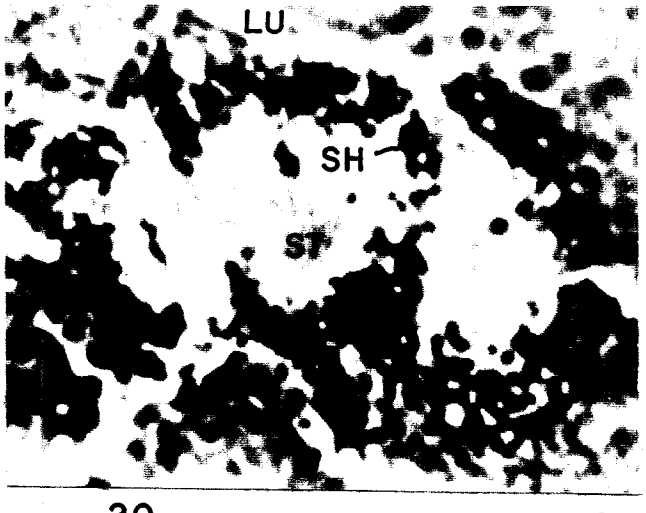
40 µm

4



100 µm

5



20 µm

6

Stage II (Maturing I) : In this stage, the connective tissue stroma which was predominant in the previous stage is reduced and the entire testis is filled with primordial germ cells and spermatogonia arranged in the form of seminiferous lobules. Each seminiferous lobule is surrounded by stromal cells. In the transverse section, the lobules seem to be arranged in a radiating manner from the middle region of each testis. Within the lobule, isolated somatic cells are found in close association with the spermatogonia (Plate XIII, Figs. 2 and 3).

Stage III (Maturing II) : The histological characteristic of this stage is that the interlobular somatic cells surrounding individual seminiferous lobule become highly reduced and the lobules get filled with cysts of dividing cells. Each lobule consists of isolated groups of two or three large nucleated, light staining spermatogonia, cysts of deeply staining small spermatocytes and spermatids which appear for the first time. (Plate XIII, Fig. 4).

Stage IV (Mature) : As the testes gradually attain maturity, the seminferous cysts within the lobules show active spermatogenesis. Most of the cysts are now filled with spermatocytes, spermatids

and spermatozoa. A few peripheral spermatogonia are also seen. The spermatozoa are arranged in a characteristic 'parachute - like' arrangement with the tails facing the centre and the heads directed towards the periphery of the cyst. A small intralobular lumen also begins to develop outside the cysts. Some spermatozoa are also released into this lumen (Plate XIII, Figs. 5 and 6).

Stage V (Oozing) : At this stage the lumen of the seminiferous lobule is very much enlarged and filled with sperms. It becomes continuous with the lumen of the vas efferens (Plate VI, Fig. 2). The interlobular somatic tissue is highly reduced. A few cysts containing residual spermatocytes and spermatogonia are found along the lobular wall. The sperms are no longer arranged in clusters. They exhibit streaming movement within the lobular space and the sperm ducts (Plate XIV, Fig. 1).

Stage VI (Spent) : The lumen of the seminiferous lobules contain large empty spaces and some degenerating residual sperms. The interlobular somatic tissue is well developed. The outer margin of the testis is crenulated (Plate XIV, Figs. 2 and 3).

PLATE XIV

Fig. 1. Semithin transverse section of the fifth stage testis of Mugil cephalus showing lumen of the seminiferous lobules, filled with sperms. LU=Lumen of the seminiferous lobule; SE= Seminiferous cyst; SL= Seminiferous lobule; IT= Interlobular somatic tissue. Methylene blue - Azure II/Basic Fuchsin.

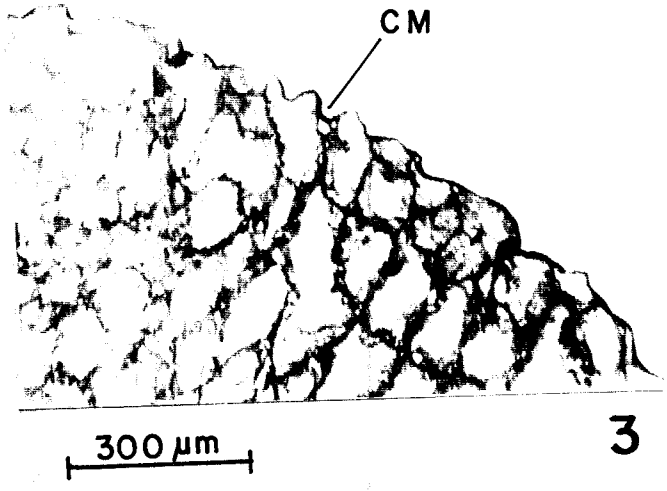
Fig. 2. Transverse section of the sixth stage testis of Mugil cephalus showing spent condition. IT= Interlobular somatic tissue. LU= Lumen of the seminiferous lobule. Lendrum's haematoxylin eosin.

Fig. 3. Transverse section of the spent testis of Mugil cephalus showing crenulated margin (CM). Lendrum's haematoxylin and eosin.

PLATE XIV

1

2



3

L. parsia

The histological characteristics of the testes of L. parsia are found to be similar to those of M. cephalus. A brief description of the salient features is as follows:

Stage I (Immature) : Characterised by clusters of densely packed, large, irregular primordial germ cells, distributed throughout the entire testis (Plate XV, Fig. 1).

Stage II (Maturing I) : Well defined seminiferous lobules with large spermatogonia occupy the major part of the testis. In some lobules, seminiferous cysts containing dividing spermatocytes are also seen. Towards the end of this stage, a few spermatids are also formed (Plate, XV, Figs 2 and 3).

Stage III (Maturing II) : The seminiferous lobules of the third stage are characterised by the presence of large number of spermatogonia. Some of the lobules show distinct seminiferous cysts with spermatocytes and spermatids (Plate XV, Fig. 4).

Stage IV (Mature) : Majority of the seminiferous lobules consist of compactly arranged seminiferous cysts of actively dividing spermatocytes, spermatids and spermatozoa. Only few isolated spermatogonia are present along the periphery of the lobules.

An intralobular lumen is seen in some seminiferous lobules. The spermatozoa exhibit the 'parachute-like' arrangement in some regions, while in others they are liberated into the lumen of the seminiferous lobule (Plate XVI Figs. 1 to 4 and Plate XVII Figs. 1 and 2).

Stage V (Oozing) : Seminiferous cysts are very much reduced in size and number. They are found along the periphery of the seminiferous lobules. The lumen of each seminiferous lobule becomes enlarged and continuous with the lumen of the vas efferens (Plate IX, Fig. 1). The entire lumen is filled with free, motile sperms (Plate XVII, Fig. 3).

Stage VI (Spent) : A few degenerating residual spermatozoa are found in the lumen of some of the seminiferous lobules. In most cases the lobules consist of empty spaces lined by seminiferous cysts of developing spermatocytes (Plate XVII, Figs. 4 to 6).

Remarks

Studies on the seasonal fluctuation in the gross structure of the male gonad and the corresponding histological changes have been made by Turner (1919) in

PLATE XVI

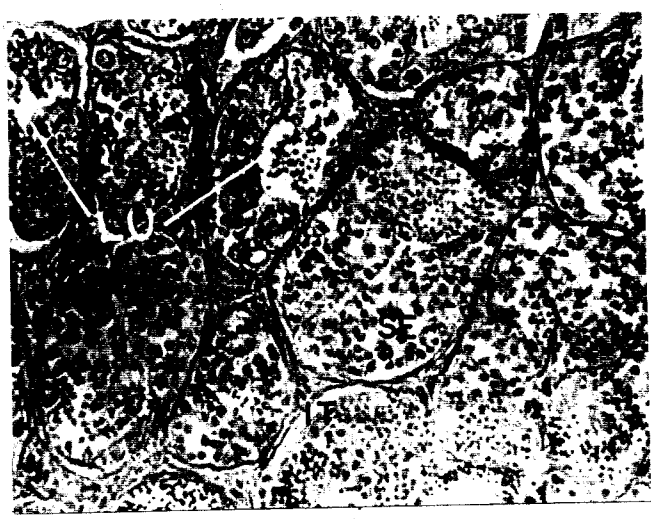
Fig. 1. Semithin transverse section of the middle part of the fourth stage testis of Liza parsia showing seminiferous lobules (SL). IT= Interlobular somatic tissue; LU= Lumen of the seminiferous lobule; SF= Seminiferous cyst. Methylene blue - Azure II/Basic fuchsin.

Fig. 2. Seminiferous lobule of the fourth stage testis of Liza parsia showing compactly arranged cysts of spermatocytes (SC) and spermatids (SD). The intralobular space contains spermatozoa (SZ). Arrow indicates spermatogonia. Harris' haematoxylin/eosin.

Fig. 3. Seminiferous cyst of Liza parsia (enlarged). IT= Interlobular somatic tissue; SC= spermatocytes ; SZ= Spermatozoa. Harris' haematoxylin and eosin.

Fig. 4. Semithin transverse section of the fourth stage testis of Liza parsia showing the different cell types. SC=Spermatocyte; SD= Spermatid and SG=Spermatogonia. Methylene blue - Azure II/Basic fuchsin.

PLATE XVI



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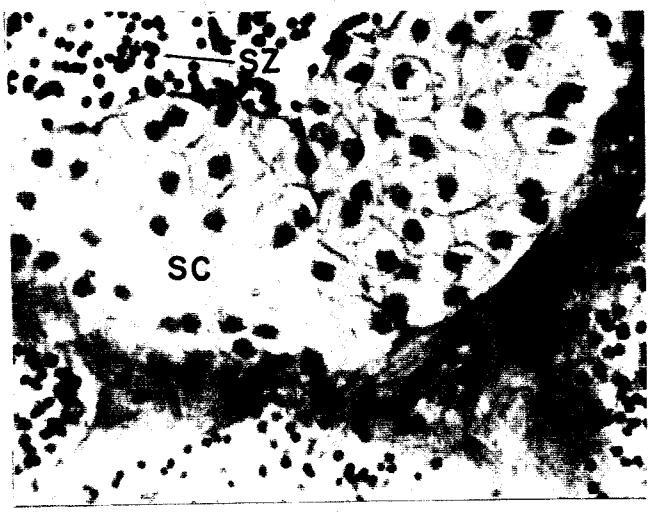

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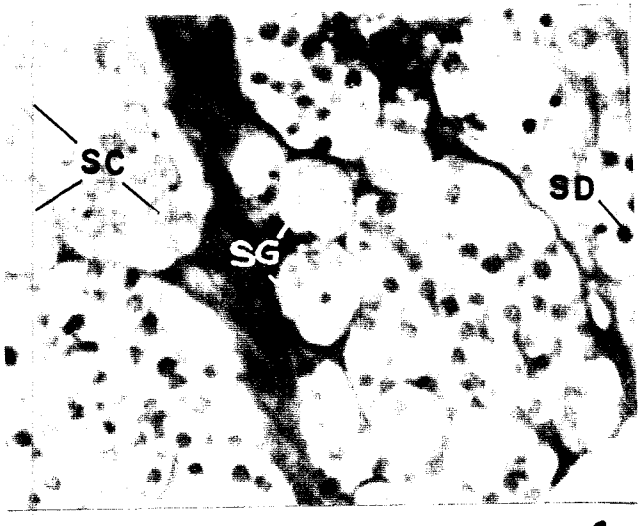

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4

P. flavescens, Craig-Bennett (1930) in Gasterosteus aculeatus, Bennington (1936) in Betta splendens, Mathews (1938) in Fundulus heteroclitus, Bullough (1939) in Phoxinus laevis, Jones (1940) in Salmo salar, Fredrick (1941) in Galeichthys felis and Weisel (1943) in Oncorhynchus nerka.

Turner (1919) observed that the weight and volume of the testes in the perch, increased with the progressive formation of spermatozoa and decreased after their expulsion during spawning. Later workers made use of these seasonal changes of the gonad to identify the different maturity stages. Thus James (1946) distinguished six maturity stages based on the macroscopic and microscopic observations of the ovary and testes in Lepomis macrochirus and Huro salmoides.

Stenger (1959) described the reproductive tissue of M. cephalus of both sexes, in different size groups of fishes. However he did not make an attempt to identify the maturity stages or give their corresponding characteristic features. Luther (1963) followed a six point maturity scale to describe the maturity stages in L. macrolepis. A similar scale was also used by Natarajan and Reddy (1980) to describe the maturity stages in L. dussumeri.

PLATE XVII

Fig. 1. Longitudinal section of a single seminiferous lobule of fourth stage testis of Liza parsia showing parachute like arrangements of spermatozoa. LU=Lumen of the seminiferous lobule; SD=Spermatid; SH=Sperm head; ST=Sperm tail; SZ=Spermatozoa; Arrow= boundary cell. Heidenhain's haematoxylin and eosin.

Fig. 2. Longitudinal section of the seminiferous lobule showing liberation of sperms into the lumen. IT=Interlobular somatic tissue; LU=Lumen of the seminiferous lobule; SZ=Spermatozoa. Mallory's triple.

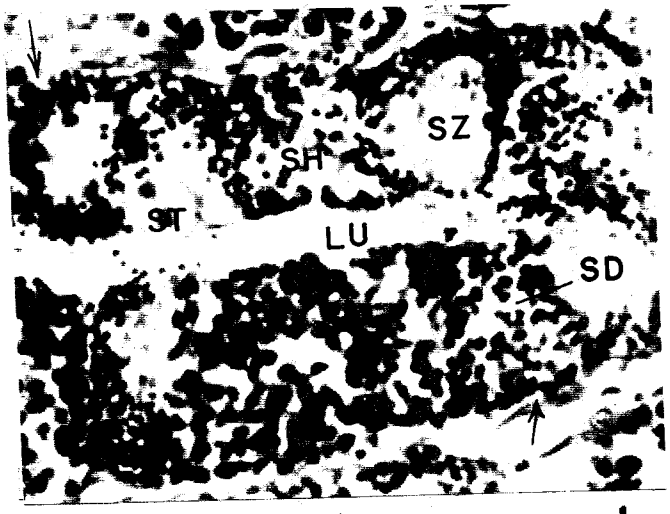
Fig. 3. Longitudinal section of the fifth stage testis of Liza parsia showing vas deferens and vas efferens filled with spermatozoa. VD= vas deferens; VE=vas efferens. Harris' haematoxylin and eosin.

Fig. 4. Transverse section of the sixth stage testis of Liza parsia showing a seminiferous lobule with a few sperms in the lumen. LU=Lumen of the seminiferous lobule; SC= spermatogenic cyst; SZ=spermatozoa (residual). Heidenhain's haematoxylin and eosin.

Fig. 5. Longitudinal section of the seminiferous lobule with degenerating residual sperms in the lumen (SZ). Heidenhain's haematoxylin and eosin.

Fig. 6. Seminiferous lobule of the spent testis Liza parsia (enlarged) showing residual spermatosoa (SZ). Heidenhain's haematoxylin and eosin.

PLATE XVII



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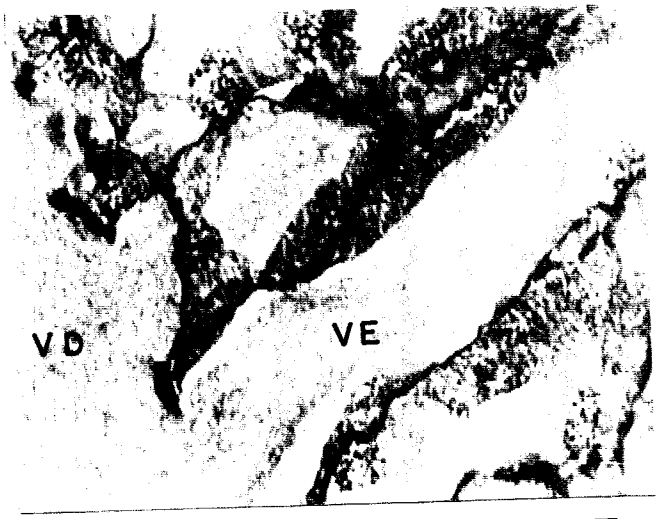

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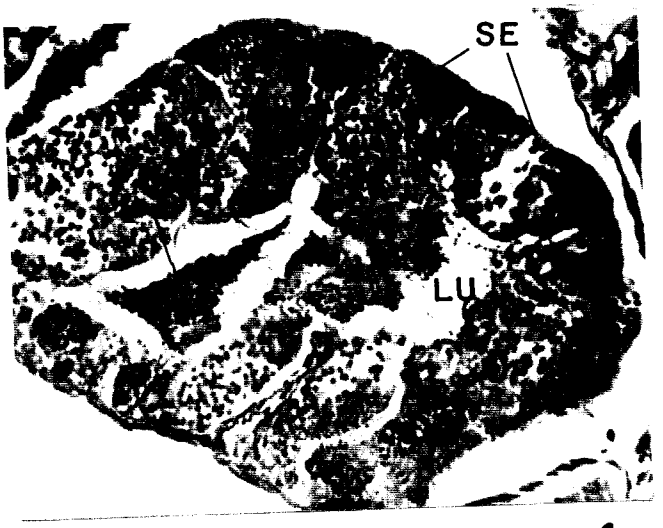

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6

As per the FAO fisheries technical paper (1974), an eight point maturity scale is recommended for total spawners. However, in the present study, the six point maturity scale described by Luther (1963) was found to be more suitable and hence it was adopted.

The testes of M. cephalus and L. parsia show distinct changes in external and internal features during the reproductive cycle. The six stages of maturity are identified taking all the morphological and histological features into consideration. All the six stages can be easily distinguished both macroscopically and microscopically. The characteristics of the stages of maturity are almost alike in both the species, except for a few minor differences.

The changes observed in the interlobular somatic tissue during the process of maturation are more prominant in the developing testes of M. cephalus as compared to that of L. parsia. In M. cephalus, during active spermatogenesis (Stage III and IV) most of the spermatogenic cysts contain cells in the same stage of development, while in L. parsia though each spermatogenic cyst consists of germ cells in the same stage of development, the number of cysts in the same phase of development are few.

In the oozing stage in M. cephalus very few cysts are seen lining the wall of the seminiferous lobule. Most of the cysts containing dormant spermatogonia are seen at the blind end of the lobule. In the oozing L. parsia on the other hand, residual seminiferous cysts are evenly distributed throughout the entire length of the lobular wall.

On the basis of the observations made in the present study, the process of maturation and the maturity cycle in M. cephalus and L. parsia can be summed up as follows. The testicular differentiation begins with the formation of isolated groups of primordial germ cells, which undergo cell division and organise themselves into distinct lobules. Gradually with the development of the testes and the proliferation of the spermatogenic cysts, the intralobular lumen increases in size and becomes continuous with the lumen of the vasa efferentia which in turn are continuous with the lumen of the vas deferens. Once the mature sperms are formed, they are liberated into the lumen from where they are released into the external medium during spawning. The spent testes, resorb the residual sperms and undergo a period of rest and recovery during which the testes regain the status of the second stage of maturity. After a short time lapse, proliferation of the dormant spermatogonia starts, marking the beginning of the next cycle of spermatogenesis.

CHAPTER V

REPRODUCTIVE CYCLE AND ENVIRONMENTAL FACTORS

In fishes, reproduction like any other physiological process, follows a cyclic pattern, the periodicity and the timing of which is under the dual control of endogenous and exogenous factors. Detailed reviews on the subject have been given by de Vlaming (1972a, 1974), Billard *et al.* (1978), Scott (1979), Wootton (1982), Lam (1983) and Bye (1984).

One of the major difficulties in studying the exact nature of the control mechanisms governing reproduction is that reproductive cycles are adaptive and each species has evolved a unique pattern, depending on its evolutionary and ecological niche. The method by which each reproductive cycle is synchronised with the environmental cycle is also species specific. Hence although reproductive timings of many species have been studied, it is not possible to use the results obtained in one species for another.

A perusal of the literature reveals that the pattern of gonadial maturation, breeding season and timing of breeding in grey mullets vary from species to species and from place to place. This is perhaps due to their wide geographical distribution and successful adaptation to

different ecological niches. For the same reason the environmental factors which influence or favour the maturation and breeding of one population of a species at a region are found to be quite different for another population of the same species inhabiting different regions. A comprehensive account of spawning periods of different species of mullets at different localities and the environmental factors influencing these activities has been given by Brusle (1981a) and Nash and Koningsberger (1981).

Information on the spawning season and time of different species of mullets in the Indian Ocean region is limited. In the tropics and subtropics, M. cephalus is reported to breed in winter (Nash and Koningsberger, 1981). In India, most of the species of mullets are known to have a prolonged breeding season, but normally spawn during the south-west monsoon. It is also recorded that generally the fry and fingerlings of mullets (the occurrence of which indicates the spawning season) are available more or less throughout the year at some locality or the other along the coast. However, the period of the peak abundance is found to vary from place to place. Sarojini (1957, 1958) has observed that M. parsia spawns from January to March and M. cunnesius from May onwards, in Bengal waters. Kurup and Samuel (1983) working on the spawning biology of L. parsia from Cochin waters have reported the peak spawning of the species from October to May. Recently

Sulochanamma et al. (1980) have found that males of M. cephalus mature earlier than females and breed during September - April in Porto Novo waters. In females, though the breeding season is seen from September to April, spawning peak is observed during October to January.

Most of the observations on the reproductive biology of mullets, relate to the females of the species. There is only limited information on these aspects in males. In view of this and since the environmental factors affect the qualitative as well as the quantitative components of reproduction and its physiology, an attempt is made here to study the breeding season and reproductive cycle of male M. cephalus and L. parsia of the Cochin waters, along with the natural environmental factors that influence the reproduction. The author is aware that this aspect is not connected directly with the title of the thesis, nevertheless an understanding of the complexity of reproduction and environmental factors is an essential prerequisite to appreciate the reproductive physiological strategy of the fish.

OBSERVATIONS

Seasonal distribution of maturity stages and breeding season.

The month-wise distribution of different maturity stages of male M. cephalus from the Cochin waters is given

in Table 1 and Plate XVIIIA. From the available data, it is clear that mature specimens are met with only in the months of November - December and May - June and spent fishes are found in the months of June, July and December, indicating that these are the months during which, spawning is likely to occur. First and second stages of maturity dominate all the other months, from July to October and January to March. Third stage fishes are observed only in a small percentage in the months of November and May.

The percentage occurrence of maturity stages of male L. parsia during the period 1982-83 is given in Table 2 and Plate XVIII B. The data reveal that the maximum number of immature specimens are met with during June-July and August, while the maximum number of oozers are met with during the period from October to May.

Condition factor 'K'

The changes in the condition factor in the males of both the species are presented in the Table 3 and Plate XIX. In M. cephalus the value of the condition factor was found to fluctuate between 0.832 and 1.242 with relatively higher values from July to September, November - December, February and April - May. In L. parsia the maximum value of the condition factor (1.277) was observed in the month of August 1982 and minimum (0.813) in July 1982. Higher 'K' values in this species were observed

TABLE - 1. Percentage distribution of maturity stages of male *M. cephalus* from the Cochin Bar mouth region, during 1982-83.

Month	No. of fish	Maturity stages					
		I	II	III	IV	V	VI
June	11	-	-	-	27.27	63.63	9.09
July	14	64.28	35.71	-	-	-	-
Aug.	13	46.15	53.84	-	-	-	-
Sept.	12	83.34	16.66	-	-	-	-
Oct.	9	77.77	22.22	-	-	-	-
Nov.	12	-	-	8.33	66.66	25.00	-
Dec.	8	-	-	-	-	25.00	75.00
Jan.	6	33.33	66.66	-	-	-	-
Feb.	15	73.34	26.66	-	-	-	-
Mar.	9	33.33	66.66	-	-	-	-
April	7	42.85	57.14	-	-	-	-
May	20	-	-	25.00	15.00	60.00	-
June	12	-	-	-	33.33	50.00	16.66
July	9	55.55	33.33	-	-	-	11.11
Aug.	8	37.50	62.50	-	-	-	-
Sept.	15	80.00	20.00	-	-	-	-
Oct.	10	60.00	40.00	-	-	-	-
Nov.	14	-	-	-	57.14	42.85	-
Dec.	7	-	-	-	-	71.43	28.57

TABLE - 2. Percentage distribution of maturity stages of male *L. parsia* from the Cochin Bar mouth region during 1982-83.

Month	No. of Fish	Maturity stages					
		I	II	III	IV	V	VI
June	20	45.00	10.00	15.00	20.00	10.00	-
July	31	38.70	12.90	12.90	26.12	9.67	9.67
Aug.	29	24.13	20.68	31.03	6.89	10.34	6.89
Sept.	22	9.09	27.27	22.72	27.27	9.09	4.54
Oct.	26	7.69	11.53	15.38	30.76	23.07	11.53
Nov.	23	-	8.69	13.04	30.43	39.13	8.69
Dec.	28	10.71	3.57	3.57	28.57	39.28	14.28
Jan.	30	3.33	6.66	10.00	33.33	43.33	3.33
Feb.	22	4.54	9.09	13.63	31.81	40.90	-
March	28	10.71	10.71	14.28	21.42	35.71	7.14
April	26	7.69	7.69	15.38	26.92	34.61	7.69
May	23	17.39	8.69	13.04	21.73	26.08	13.04
June	28	21.42	25.00	17.85	21.42	10.71	3.57
July	30	23.33	16.66	20.00	26.66	13.33	-
Aug.	23	21.73	21.73	8.69	21.73	17.39	8.69
Sept.	28	21.42	28.57	25.00	14.28	7.14	3.57
Oct.	30	13.33	20.00	10.00	30.00	13.33	13.33
Nov.	33	6.06	6.06	18.18	24.24	36.36	9.09
Dec.	32	12.50	9.37	15.62	18.75	25.00	18.75

PLATE XVIII

Monthly variation in the percentage composition of the different maturity stages in the males of (a) Mugil cephalus (b) Liza parsia.

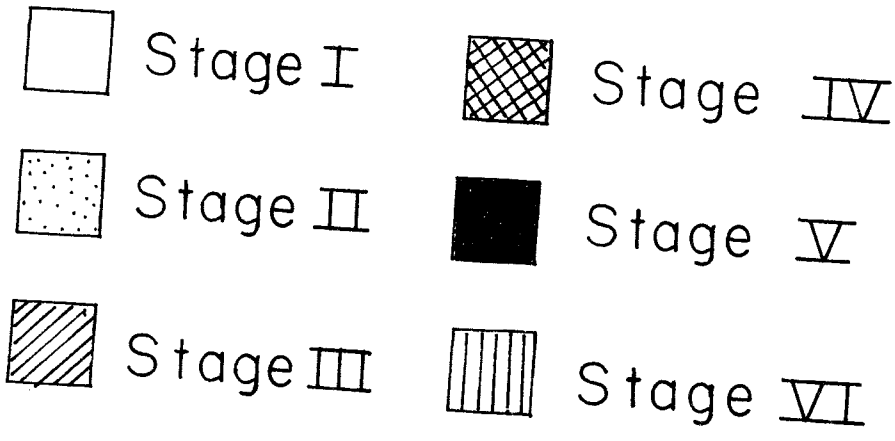


PLATE XVIII

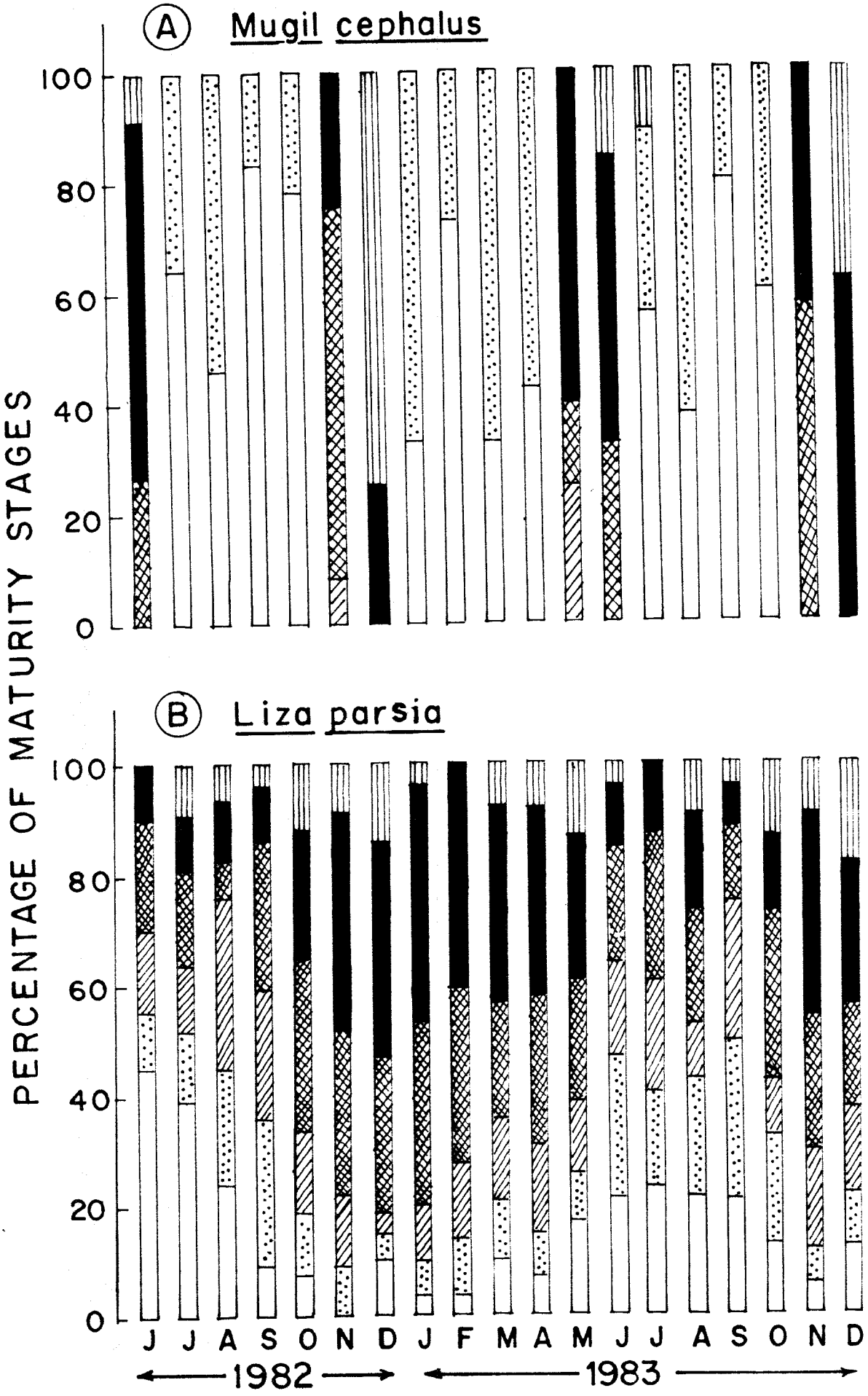

A Mugil cephalus
B Liza parsia
PERCENTAGE OF MATURITY STAGES
100
80
60
40
20
0
J J A S O N D J F M A M J J A S O N D
1982
1983

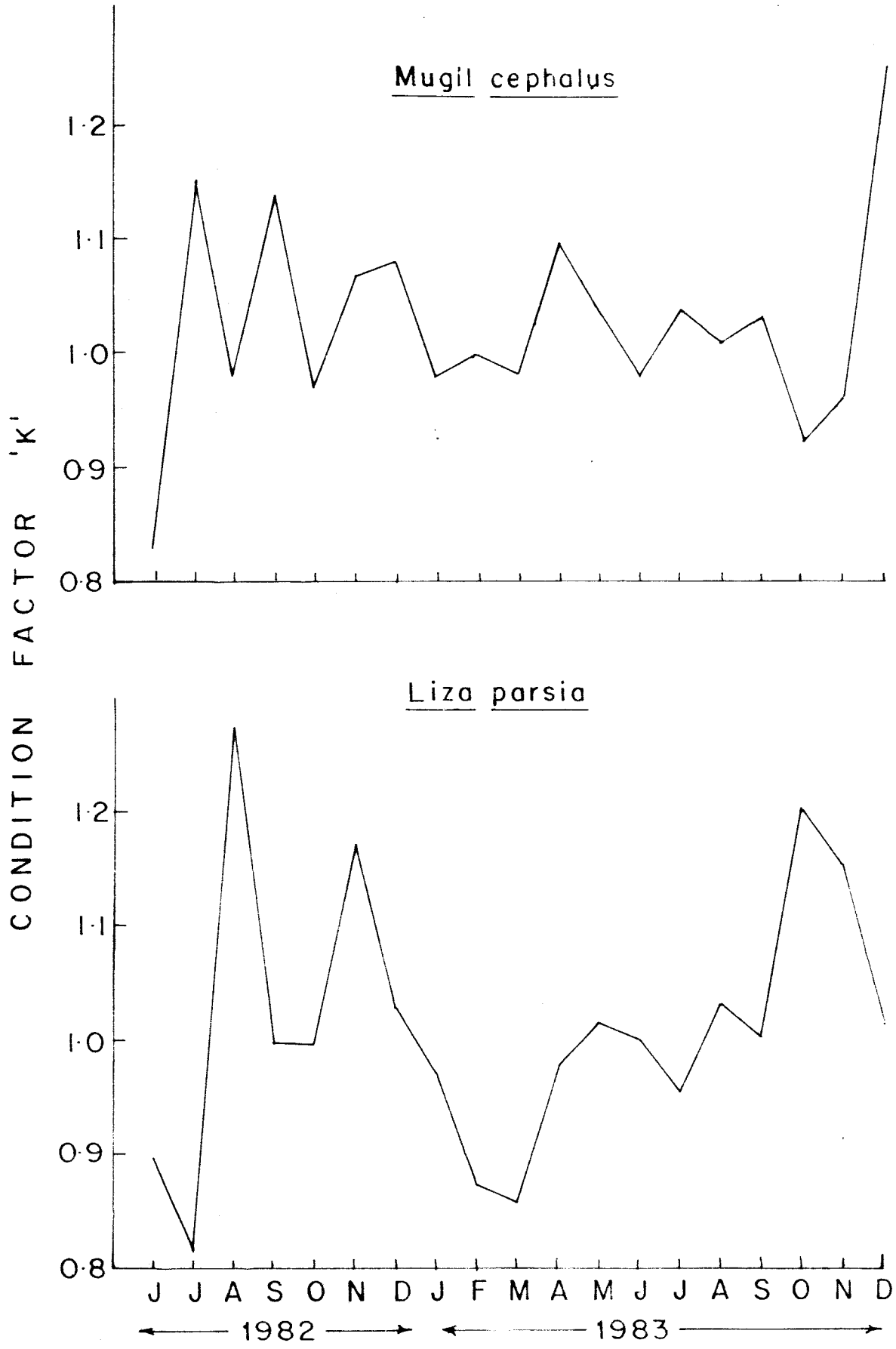
TABLE - 3. Condition factor 'K' values for male *M. cephalus* and *L. parsia* collected from the Cochin Bar mouth during 1982-83

Months	Condition factor 'K'	
	M. cephalus	*L. parsia*
June 1982	0.832	0.895
July	1.153	0.813
August	0.979	1.277
September	1.140	0.974
October	0.977	0.995
November	1.068	1.172
December	1.089	1.03
January 1983	0.982	0.969
February	1.010	0.870
March	0.986	0.857
April	1.095	0.978
May	1.038	1.016
June	0.980	1.008
July	1.043	0.952
August	1.012	1.035
September	1.030	1.002
October	0.926	1.208
November	0.966	1.152
December	1.242	1.014

PLATE XIX

Variation in the condition factor of male *Mugil cephalus* and *Liza parsia* of the Cochin area from June 1982 to December 1983.

PLATE XIX


Mugil cephalus
Liza parsia
CONDITION FACTOR 'K'
1·2
1·1
1·0
0·9
0·8
J J A S O N D J F M A M J J A S O N D
1982
1983

during May - June and August to December. Comparatively low values were found in July, February and March.

Gonado-Somatic Index (GSI):

The monthly distribution of the GSI of M. Cephalus and L. parsia from June 1982 to December 1983 in the Cochin waters is given in Table 4. In M. Cephalus the GSI was found to vary from 0.024 to 1.176. Relatively higher GSI was recorded in May-June and November, indicating intense gonadal activity. In the case of L. parsia, the GSI was seen varying between 0.312 and 1.422 in different months. However, the higher values of GSI recorded during October to May suggest that the species has a prolonged breeding period in Cochin waters with intense breeding activity from December to February.

Environmental factors

Month-wise distribution of the surface water temperature, salinity and dissolved oxygen of the Cochin Bar mouth region and mean day length are given in Table 4 and depicted in(Plate XX, Figs. A-C).

The average monthly surface water temperature of the Cochin back waters from where the collection was made, was found to fluctuate between 27°C (January 1982) and 32.2°C (May 1983). The fall in temperature in January

TABLE - 4. Monthly distribution of GSI of male *Mugil cephalus* and *Liza parsia* and environmental parameters from the Cochin Bar mouth region.

Month	Mean GSI		Mean Day length		Mean Temperature (°C)	Mean Salinity (ppt)	Mean dissolved oxygen (ml/l)
	M. cephalus	*L. parsia*	Hrs.	Min.			
June '82	1.176 ± 0.330	0.351 ± 0.046	12	22	30.2	34.215	5.354
July	0.026 ± 0.002	0.582 ± 0.021	12	45	29.5	17.318	6.813
Aug.	0.035 ± 0.005	0.613 ± 0.411	12	23	30.0	10.157	7.312
Sept.	0.037 ± 0.018	0.710 ± 0.396	12	07	31.0	21.307	3.490
Oct.	0.233 ± 0.011	0.880 ± 0.187	11	51	30.0	14.328	4.454
Nov.	0.888 ± 0.244	1.142 ± 0.227	11	38	31.0	20.473	3.852
Dec.	0.114 ± 0.108	1.374 ± 0.448	11	33	30.0	29.940	2.768
Jan. '83	0.144 ± 0.014	1.422 ± 0.553	11	39	27.0	33.291	3.009
Feb.	0.028 ± 0.072	1.331 ± 0.061	11	48	30.0	33.291	4.620
Mar.	0.029 ± 0.009	1.261 ± 0.312	12	03	32.0	35.314	3.969
April	0.212 ± 0.204	1.112 ± 0.251	12	20	31.0	35.567	4.313
May	1.015 ± 0.246	1.031 ± 0.519	12	34	32.2	36.575	3.716
June	0.815 ± 0.367	0.402 ± 0.238	12	41	30.0	35.000	5.484
July	0.024 ± 0.007	0.312 ± 0.012	12	42	28.0	14.730	7.102
Aug.	0.042 ± 0.011	0.451 ± 0.035	12	33	29.0	5.385	6.379
Sept.	0.027 ± 0.004	0.513 ± 0.233	12	04	30.0	9.345	8.159
Oct.	0.040 ± 0.002	0.784 ± 0.205	11	52	29.0	18.306	6.387
Nov.	1.122 ± 0.401	1.180 ± 0.417	11	37	30.5	26.119	4.942
Dec.	0.274 ± 0.140	1.291 ± 0.523	11	33	30.0	28.312	3.432

PLATE XX

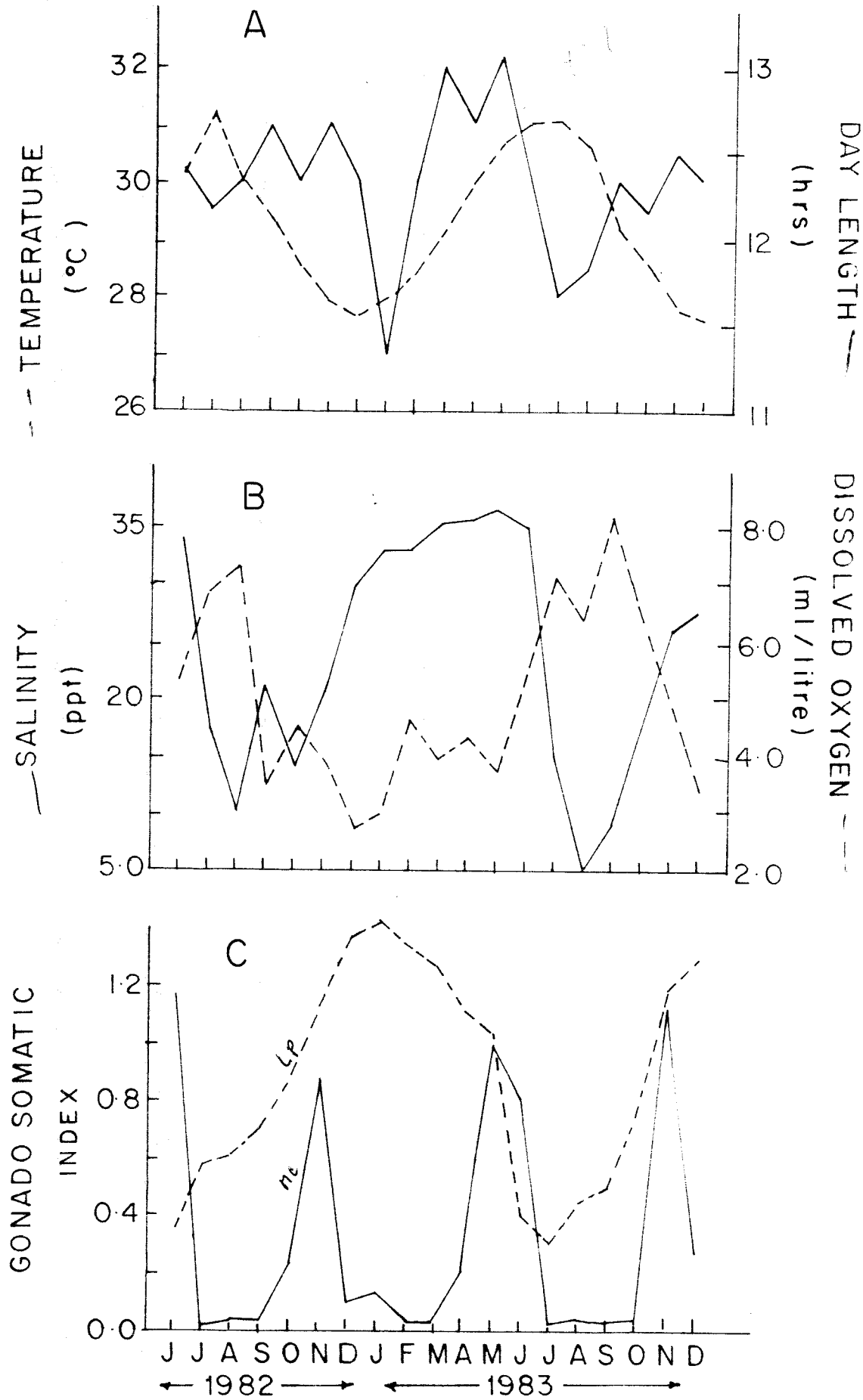
Monthly distribution of temperature and daylength, salinity and dissolved oxygen and the GSI of male *Mugil cephalus* and *Liza parsia* of the Cochin Bar mouth area during June 1982 to December 1983.

A Temperature (– – –) ; Day length (———)

B Salinity (———) ; Dissolved oxygen (— — —)

C GSI , *M. cephalus* (———) ; GSI, *L. parsia* (– – –)

PLATE XX


A
32
30
28
26
TEMPERATURE (°C)
13
12
11
DAY LENGTH (hrs)
B
35
20
5·0
SALINITY (ppt)
8·0
6·0
4·0
2·0
DISSOLVED OXYGEN (ml/litre)
C
1·2
0·8
0·4
0·0
GONADO SOMATIC INDEX
Lp
nc
J J A S O N D J F M A M J J A S O N D
1982
1983

appears to be due to the winter season while that observed in July (28°C), due to the onset of monsoon.

The salinity values were high (35.314 ppt to 36.575 ppt) during March-May, the highest being recorded in May 1983. From July onwards the salinity declined, to reach the minimum (5.385 ppt) in August 1983. After monsoon, the salinity of the water was seen increasing from October onwards.

The average monthly dissolved oxygen of the surface water varied from 2.768 to 8.159 ml/l. The highest value was recorded in September 1983 and the lowest in December 1982. The dissolved oxygen content was found to be relatively higher in the months of July and August in both the years, indicating the effect of the monsoon waters.

The mean day length for the different months is given in the Table 4. Shorter days are recorded from October (11 hrs. 51 mins.) to February (11 hrs. 48 mins.) and longer days from March(12 hrs. 3 mins.) to July (12 hrs. 42 mins.). From August onwards, a gradual decrease in the day length is recorded with the shortest days being recorded in the month of December (11 hrs. 33 mins.).

A comparison of the distribution of the GSI values and environmental parameters indicate that one of the peaks of GSI observed in May-June occurs when salinity and temperature start decreasing from the highest values recorded for the season. The second peak seen in November coincides with the period when these parameters are increasing from the lowest recorded during the rainy season. Similarly, the peak GSI values recorded in May-June occur during the maximum day length period and the other peak observed in Nov-Dec. during the minimum day length. In L. parsia, the highest GSI values observed during November to May, coincide with the period when both salinity and temperature of the surface water gradually increase. Similarly the photo-period during this period was also observed to increase gradually as the season advanced from November to May.

In respect of condition factor, although there is no clear and distinct relationship between this parameter and the peak spawning season in both the species, in M. cephalus 'K' values were found relatively higher during the two peak breeding periods in the male.

DISCUSSION

A perusal of the various reports available on the maturity and spawning of mullets of different regions of

India indicate that there is no uniformity in respect of the time of spawning at different places. The observations in this aspect by different authors are inconsistent. However the monthly distribution of the maturity stages of the males of M. cephalus from the Cochin waters shows two specific periods of availability (May - June and Nov - Dec) of oozing and spent specimens in a year. This suggests the probability of two spawning periods in the local population. This observation is in agreement with those of Stenger (1959) who, from histological study of the gonads concluded that there was a possibility of M. cephalus off the coast of Florida, spawning more than once a year. Two periods of spawning of M. cephalus in the vicinity of Hong Kong is also reported by Bromhall (1954).

In L. parsia a single protracted period of relatively high percentage of mature and oozing stages are met with from October to May indicating an extended period of spawning. The presence of spent fishes throughout this period further supports this inference. This observation is in agreement with the study made in 1979 by Kurup and Samuel (Kurup and Samuel, 1983). Sarojini (1957, 1958) has reported the spawning of M. parsia from January to March. She has also recorded two groups of developing ova in the ovaries of M. parsia, ripening about two months apart (Sarojini, 1957). Such distinct

demarcation, in the recrudescence of the male gonad was not found among the specimens observed from October to May.

The coefficient of condition or the condition factor (K) is used widely to express the relative robustness of the fish, suitability of an environment for a species as well as to measure the effect of environmental improvement and attainment of sexual maturity and spawning (Luther, 1963). The fluctuations observed in the condition factor of fishes have been attributed to different reasons (Hickling, 1945; Qasim, 1957). While Venkatramani (1979) working on carangid fishes of Porto Novo waters reported that changes in the relative condition factor reflected the spawning cycle in the fish, Thomas (1969) from his studies on Upeneus tragula concluded that the changes in the condition factor did not appear to be related to either the sexual cycle or the feeding intensity but to some other factors.

The coefficient of condition of Indian mullets have been studied by Sarojini (1958), Luther (1963), Patnaik (1962) and Rangaswamy (1976). A direct relation is found between the feeding intensity and fluctuation in the relative condition factor in L. macrolepis and M. cephalus collected from the Mandapam Lagoon (Luther, 1963).

In the present study, the condition factor of male M. cephalus collected from the Cochin bar mouth region, does not indicate any specific pattern of change, with respect to the maturity cycle. High 'K' values were observed in the month of July, September and April, when the majority of the fishes were in the immature or early maturity stage, as well as in the months of November, December and May when the fishes were in the mature and oozing stages. This indicates that there is no perceptible effect of the breeding cycle on the condition factor, although during the peak breeding of males, the 'K' values were relatively higher.

In Liza parsia a distinct peak in the value of 'K' is observed in August 1982. Two more peaks are recorded in the months of November 1982 and Oct-Nov 1983. During the rest of the year, relatively low 'K' values are observed. It is speculated that the high values in the month of November in both the years, might be due to the presence of mature gonads, that mark the beginning of the spawning season. The subsequent fall in the 'K' values might be due to the continued spawning activity. The fact that high values of coefficient of condition are also observed in the month of August 1982 suggests that in L. parsia the condition factor is influenced not only by the breeding cycle but also by some other ecophysiological factors. A similar case is reported by Kurup (1982) in

Daysciaena albida where, relatively high 'Kn' values are observed both in the spawning and the non-spawning seasons.

As compared to the temperate species that exhibit seasonal reproductive cycles with precise timings, the tropical fishes have generally protracted cycles. This is because in the temperate regions, there is a distinct seasonality in the fluctuation of environmental parameters, where as in the tropics, seasons merge with one another, restricting the fluctuation in the environmental parameters to a marginal range. A study of the major environmental parameters of the Cochin backwaters during the period 1982-83 revealed very little fluctuation in the mean day length and temperature. However, the dissolved oxygen content and salinity varied over a relatively wide range during the period of the study. The hydrological parameters observed during the course of the present study, agree with those recorded by earlier workers (George and Kartha, 1963; Ramamritham and Jayaraman, 1963).

Among the exogenous factors that affect the reproductive cycle, temperature and photoperiod seem to be the most important ones. Earlier, photoperiod was considered to be the more important factor, but further studies revealed that photoperiod was not independent of temperature in nature and that when one was isolated from the other, the results obtained could be

quite misleading. For e.g., work done on catfishes by Sehagal and Sunderaraj (1970) and Sunderaraj and Sehgal (1970) indicated that when constant temperature and varying photoperiod was used, the catfish reproductive cycle seemed to be primarily timed by photoperiodic responses. Further work with changing temperatures showed that, although the catfish could use photoperiodic cues these were unimportant under natural conditions and that the main factor regulating the reproductive cycle was water temperature (Vasal and Sunderaraj, 1976).

Among the temperate species, gonadal recrudescence seems to be stimulated by long photoperiods in combination with warm temperature in the spring spawners as observed in Notropis bifrenatus (Harrington, 1950, 1957); Gasterosteus aculeatus (Baggerman, 1957, 1972, 1980) and Oryzias latipes (Yoshioka, 1962, 1963). Short or decreasing photoperiods are found to induce the same changes among the autumn spawners as exemplified by Salmonids (Combs et al, 1959; Billard et al., 1981; Breton and Billard, 1977; Shiraishi and Fukuda, 1966 and Henderson, 1963). However, in the tropical species of Oryzias, namely, O. javanicus, varying photoperiods at ambient temperature (27±1°C) do not seem to affect the pattern of spawning frequency or the fecundity while a temperate species of the same genus, O. latipes, seems to be sensitive to photoperiodic changes (Lam 1983).

Temperature and light intensity were also identified as the important cues for seasonal peaks of gonadal activity in Tilapia laucosticta (Hyder, 1970). Higher light intensities were found to delay sexual maturity in both sexes of Tilapia zilli while increase in the temperature up to 28-31°C, increased the reproductive rate of T. mossambica (Mironova, 1977). In the wild guppy, photoperiods ranging from continuous darkness to continuous light, did not affect ovarian development but continuous light appeared to inhibit spermatogenesis as compared to total darkness. Kuo et al. (1974) working on M. cephalus found that a constant temperature of 21°C and reduced photoperiod was very effective in inducing vitellogenesis. Kuo et al. (1974) and Kuo and Nash (1975) reported that in Hawai under natural conditions, vitellogenesis begins shortly before day length reaches its annual minimum (10 hrs and 50 mins.). Under experimental conditions they were able to stimulate vitellogenesis within 49-62 days by a constant photoperiod of 6 hours of light and 18 hours of darkness.

In the present study two distinct peaks of gonadal maturation and maximum GSI were observed in the case of M. cephalus during the months of November-December and May-June. Since the ripe stages of the gonad were observed at both times of the year when the day length was minimum (11 hrs 33 mins Nov-Dec) and also when the day length was maximum (12 hrs 49 mins June-July), it

may be concluded that gametogenic changes can take place independent of the photoperiodic rhythm. In L. parsia no sharp peaks of gonadial maturity was observed. However, the data indicates that there is a definite decrease in the GSI values during the months of July and August. The reproductive cycle is protracted with mature specimens being available almost throughout the year. Majority of the ripe specimens are found to occur from November to May within a range of minimum and maximum day length.

The role of temperature in reproductive activities and timing of breeding activities in relation to the seasonal cycle has received much attention. Studies by Mathews (1939) indicate that the final stages of maturation and spermatogenesis in Fundulus heteroclitus are governed by temperature. Lake (1967) reported the occurrance of spermiation in Plectroplites ambiguus at temperatures below 23.6°C but not ovulation. Billard et al. (1978) reported spermiation in cyprinids all the year round but ovulation only in the warm season. High temperature is known to promote spermiation in the lake chub Couesius plumbeus (Ahsan, 1966) while low temperature and decreasing photoperiod is found to initiate spermiation in rainbow trout (Breton and Billard, 1977). Sexual cycling of carps in the temperate regions seems to be greatly dependent on temperature. Gupta (1975) maintained

Cyprinus carpio at 23°C and found that 25% of the females commenced spawning at fifteen months as against four years under the natural temperature. Kausch (1975) observed that the sexually mature carps could be obtained throughout the year in the tropics and was assumed to be due to the effect of the sustained high temperature. De Vlaming (1972) demonstrated that in Gillichthys mirabilis high summer temperatures brought about gonadal regression. Similar observations were also made by Gillet et al. (1978) in Carassius auratus; Burger (1940) in Fundulus heteroclitus; Ahsan (1966) in C. plumbeus; Yaron et al. (1980) in Mirogrex terraesanctae; Kuo et al.(1974) and Kuo and Nash (1975) in M. cephalus. Abraham, 1963 (cited from Brusle 1981) noted that in Israel, oogenesis of M. cephalus and M. capito began when water temperatures are highest (27-30°C) and oocytes ripen as temperature begins to decrease. Observations of early ripening of oocytes in lake Tiberias has lead to the speculation that in warmer waters, M. cephalus may spawn twice a year (Brusle, 1981a).

A comparison of the distribution of different maturity stages and the temperature in the study area indicates that temperature has not any profound influence on the reproductive cycle under the natural conditions in both M. cephalus and L. parsia. This may be due to the relatively small range of temperature which is found to vary from 27 to 32°C. However, higher GSI was observed

either when the temperature of the surface water was seen decreasing from the maximum peak or increasing from the lower values recorded during the monsoon months. It may also be observed here that since the area chosen for the study does not exhibit great fluctuations in temperature or photoperiod, it is very likely that the reproductive cycles of the mullet population in this region might not have been timed as per environmental cues alone. It is highly probable therefore that the timing of the reproductive cycle in the mullets of the indegenous population of Cochin waters is endogenously controlled under the natural conditions.

In most teleosts salinity is regarded a less important factor in affecting gametogenesis as compared to temperature and photoperiod. In the milkfish, gametogenesis is known to occur within a wide range of salinity 7-12 ppt (Nash and Kuo, 1976; Kuo et al., 1979); 13.7 - 29.8 ppt (Liao and Chen, 1979; Tseng and Hsiao, 1979) and 28 - 35 ppt (Lacanilao and Marte, 1980). However, vitellogenesis is inhibited in fresh water. Stequert (1972) and Roblin (1980) found that in Dicentrarchus labrax spermiation could occur in salinities as low as 1 - 2 ppt while ovulation could not occur.

Though grey mullets are reported to grow to a large size in the lakes, coastal lagoons and ponds, spawning is generally not observed in these ecosystems

Heldt, 1948; Hedricks, 1961; Berg, 1965 and Abraham et al., 1966). All records on grey mullet spawning in nature show a strong preference for oceanic water as the medium of incubation. A number of species of mullets are known to exhibit spawning migrations towards the sea - M. dobulla (Thomson, 1955) M. cephalus and M. curema (Moore, 1974); M. capito (Libosvarsky and Darrag, 1975); M. cephalus (Tseng, 1975). Cervigon and Padron (1974) and Wallace (1975) observe that grey mullets that live in hypersaline conditions migrate to natural oceanic waters to spawn. Brusle (1981a) reports that specimens of M. cephalus confined to freshwater do not ovulate. Abraham et al. (1966, 1967) observe that M. cephalus females prevented from their seaward migration undergo an arrest of gametogenesis and in those confined to freshwater, oocytes develop only up to the previtellogenic stage. Further Kuo et al. (1973) report that though male M. cephalus maintained in running sea water of 32 ppt completes spermatogenesis, oocytes reach only up to the tertiary yolk globule stage. The salinity also influences the endocrine regions secreting prolactin and gonadotropin in the pituitary of Mugil (Blanco-Livni and Abraham, 1970). It was found that the gonadotropin content of the species held in freshwater is considerably lower than that of the fish from sea water and prolactin content was much higher in the freshwater species than those in the sea water.

In the present study it was seen that the high GSI values of M. cephalus (1.176 and 1.015) coincided with a salinity of 34.215 and 36.575 ppt while in L. parsia the maximum GSI range (1.142 to 1.031) was noticed from November to May when the salinity range was noticed from 20.473 to 36.575 ppt.

Though there are reports of M. cephalus males completing spermatogenesis in confined waters (Eckstein 1975), during the present study it was observed that the males of M. cephalus confined to brackish water ponds at Puthuvypeen for more than two years did not indicate a gonadal maturity beyond the second stage. Histological study of the testes of these specimens indicated the presence of only spermatogonia and a few spermatocytes.

The effect of dissolved oxygen on gametogenesis has not been studied in detail by many authors. Low dissolved oxygen is known to prevent spawning in Pimphales promelas (Brungs, 1971) and in Proximus nigromaculates (Carlson and Herman, 1978). Gillet et al. (1981) found that in temperate gold fish, low levels of dissolved oxygen caused gonadal regression. This is probably true of tropical gold fish also (Lam, 1983). Dissolved oxygen level was not considered as a limiting factor for gametogenesis in mullets. In the present

study, the value of dissolved oxygen seemed to vary between 3 and 8 ml/litre. The values of highest oxygen content were found to coincide with least GSI in M. cephalus.

In most fishes the reproductive cycle is endogenously controlled, and the environmental parameters such as temperature, salinity and pH act as cues for synchronising the reproductive rhythms with that of the environment so that the young ones can take maximum advantage of the optimum ecological conditions. But in some fishes, the endogenous rhythms seem to be independent of the environmental changes as in the case of Oryzias javanicus, where the fish spawns to brief periods daily. Alteration of photoperiod or salinity does not effect the spawning pattern of this fish. Similarly, photoperiods of different duration from continuous darkness to continuous light do not affect the sexual maturity of Sarotherodon mossambicus (Lam, 1983). A brief review of the endogenous control of reproduction is given by Bye (1984).

A specific period of time is required to complete a full cycle of events of gametogenesis in all fishes. In the temperate regions, drastic changes in the environmental conditions might directly or indirectly be responsible for maintaining this periodicity. But whether the subtle changes in the environment can be responsible for the

timing of the reproductive cycle among the tropical fish is yet to be studied comprehensively (Bye, 1984). As suggested by Schwassmann (1971, 1978) it is quite possible that, endogenous rhythms, govern the process of gametogenesis upto the final stage of maturity, while spawning is triggered by sudden environmental fluctuation (monsoon, upwelling, reduction in temperature and salinity etc.).

As the fluctuation in the environmental parameters in the study area are found to be distributed within a small range it is difficult to identify the specific effect of any single parameter on gametogenesis or spawning. Further, the environmental factors studied at present are also found not to affect significantly the reproductive cycle of the fishes. These facts indicate that the reproductive cycles of both M. cephalus and L. parsia are mainly governed by an endogenous rhythm, under natural conditions rather than the environmental factors. However, the fact that these rhythms are not independent of the environmental factors is evidenced by the observation that brackishwater pond reared specimens of two years and more, do not show normal gonadal development. The gonadal development in these fishes reach only up to the second maturity stage. These results support the view put forward by Nash and Koningsberger (1981) that reproduction in grey mullet is not a strictly controlled and regulated act.

The reason for the non-availability of spent fishes in large numbers may be perhaps due to the speedy recovery of the fish into the second stage of maturity, marking the beginning of the next cycle. Similarly the number of third stage fishes obtained were also very few. They were obtained only in the months of May and November when most of the fish were already in the fourth stage of maturity. This indicates that, third stage is of a very short duration. Second stage seems to be the one with the longest duration.

If the reproductive cycle of a species in a given area has to be experimentally altered by environmental manipulation for augmenting aquacultural returns, a knowledge of the natural cycle of the species and the corresponding environmental conditions is essential. The present work thus has some applicability in this direction. Eventhough there is no appreciable fluctuation in the environmental parameters in the natural environment of species, a slight shift from the normal environmental condition can completely upset the endogenous rhythm. As Scott (1979) has pointed out, "any recurring environmental variable within the sensory competence of the organism can act as a timing cue". The endogenous rhythm governing gametogenesis and spawning are therefore not independent of the ecological conditions, on the contrary they are highly sensitive to environmental cues and operate with normal periodicity only when the fishes are subjected to the natural conditions.

CHAPTER VI

SPERMATOGENESIS

The spermatogenesis in teleost fishes involves a series of cytological events that begin with the origin and differentiation of primordial germ cells and terminate with the release of the mature spermatozoa into the lumen of the seminiferous lobules. In the earlier works this process was studied by the standard histological and staining methods and by using light microscopy (Hann, 1927; Vaupel, 1929; Stromstein 1931; Goodrich et al., 1934; Dildine, 1936; Mathews, 1938; Jones, 1940; Weisel, 1943 and Lofts and Marshall, 1957). During the past one and half decades, however, there has been considerable expansion in the knowledge by the application of cytological techniques and electron microscopy. The different aspects of the origin and migration of the primordial germ cells, transformation of spermatids into mature spermatozoa and the cellular details of the associated somatic cells of the testis at the ultrastructural level have been studied in a number of teleost fishes - Cyprindontiform fishes : Dodone and Narbaitz (1967); Gresik et al. (1973a, 1973b); Satoh (1974); and Grier (1976); Poecilid fishes: DePelice and Rasch (1969); Billard (1970a, 1970b) and Grier (1973, 1975); Anguillid fishes: Ginsburg and Billard (1972); Billard

and Ginsburg (1973); Colak and Yamamoto (1974) and Todd (1976). The works of Mizue (1969) on black sailfin molly, of Stanley (1969) on Oligocottus maculosus, of Hurk et al.(1974a, 1974b) on Mollinesia latipinna, of Asai (1971) on Lebistes reticulatus, of Ruby and McMillan (1975) on stickle back, of Mattei and Mattei (1974, 1978a, 1978b) on Lepidoyaster and of Mattei and Mattei (1975) on Elopomorpha are also noteworthy. The various aspects of spermatogenesis have been reviewed by Mattei (1970), Grier (1981), Billard et al. (1982) and Nagahama (1983).

In Mugilidae, the investigations on spermatogenesis are limited. Among the earlier works, the cytological description of the gonadial tissue in M. cephalus by Stenger (1959) is significant. Later, the cytological characteristics of the germ cells in M. auratus, M. labrosus and M. ramada were studied by Leray (1968), Thong (1969) and Cassifour (1975). These studies, however, did not give the ultrastructure of spermatogenesis as they were entirely based on light microscopy. In 1978, Van Der Horst and Cross described the cellular structure and spermatogenesis in L. dumerilli. In the same year Brusle and Brusle (1978a, 1978b) studied the differentiation and development of early germ cells in L. auratus. Subsequently, Brusle (1980) reported the sequential cytological events leading to the formation of primary spermatocytes from primordial germ cells in M. cephalus,

and later (Brusle, 1981b) from the secondary spermatocytes to spermatozoa in the case of L. auratus.

In the present study an attempt is made to understand the intrinsic cellular changes occurring in the germ cells of M. cephalus and L. parsia during the process of transition from the primordial germ cell to the spermatozoa.

SPERMATOGENESIS IN MUGIL CEPHALUS

As in the case of other teleost fishes the germ cells of M. cephalus develop in cysts. The different stages of spermatogenesis and spermiogenesis are distinguished on the basis of the size of the cells, the nuclear characteristics, and the cytoplasmic morphology. Every mature gamete (the spermatozoon) before its formation passes through a series of cellular stages namely - the Primordial germ cell, Spermatogonia, Primary spermatocyte, Secondary spermatocyte and the Spermatid. The characteristic features of each of these stages are as follows.

Primordial germ cells

Primordial germ cells are found in large numbers in the immature and the early maturing testis. They are also seen in very few numbers in the other stages. Within

the testis they first make their appearance as small groups of compactly arranged mass of large cells distributed in the connective tissue stroma of the testis (Plate XIII, Fig. 1). The cells are irregular in outline and measure about 10 to 11.5 /um in length and 8.5 to 9 /um in width. The cell membrane is in close contact with the inner surface of the seminiferous lobule. The nucleus is large with a distinct nucleolus. In some cells more than one nucleoli are seen. The nucleus is slightly eccentric in position and measures about 6 to 6.5 /um in length and 4 to 5 /um in width. The chromatin matter is evenly distributed. The ratio of nucleus to cytoplasm (N/C) is 0.3. The cytoplasm is filled with a large number of cytoplasmic organelles (Plate XXI, Fig. 1).

Spermatogonia

Spermatogonia are the largest of the germ cells found in the male gonad (Plate XXI, Fig. 2). In the early maturity stages of the testis these cells are found in large numbers along the lobular walls. In the advanced maturity stages (Stage III, IV and V) however, they are seen only in few numbers, scattered along the seminiferous lobule and in clusters at the terminal end of the lobule. The spermatogonia are found in close association with thin walled irregular somatic cells

PLATE XXI

Fig. 1. Semithin section of the testes of Mugil cephalus showing primordial germ cell (PG). Note the nonspherical nature of the cell and nucleus. SC= Spermatocytes. Methylene blue - Azure II/ Basic fuchsin.

Fig. 2. Longitudinal section of a seminiferous lobule of Mugil cephalus (enlarged) showing spermatogonia (SG). Harris' haematoxylin and eosin.

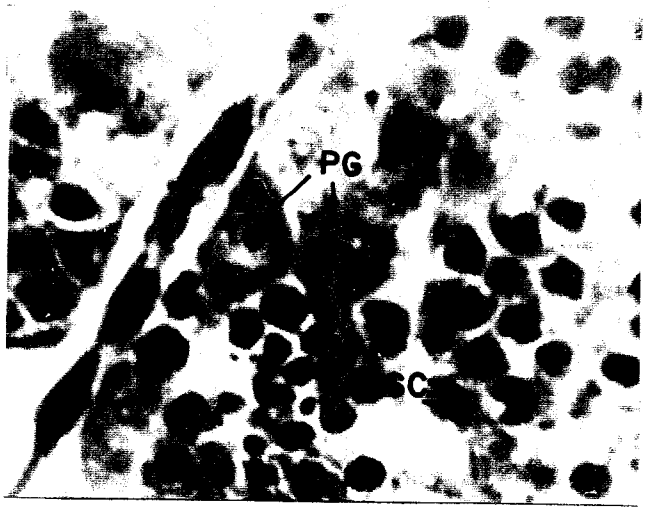
Fig. 3. Electron micrograph showing the ultrastructure of the spermatogonium of Mugil cephalus surrounded by the Sertoli cell (SR). Note the smooth out line of the nucleus, the electron dense cytoplasm and organelles. CE=Cell membrane; CY= Cytoplasm; MI= Mitochondria; NE= Nuclear envelope; NL= Nucleolus; NU= Nucleus.

Fig. 4. Ultrastructure of the spermatogonium of Mugil cephalus. Note the chromatin network (CH) and the electron dense cytoplasm (CY). CE= Cell membrane; MI= Mitochondria; NE= Nuclear envelope; NL= Nucleolus; NU= Nucleus.

Fig. 5. Electron micrograph of the primary spermatocyte of Mugil cephalus showing evenly distributed chromatin material in the nucleus and few mitochondria (MI) in the cytoplasm. Note that the electron density of the nucleus (NU) and the Cytoplasm (CY) is almost the same. CE= Cell membrane. Arrow indicates the nuclear envelope.

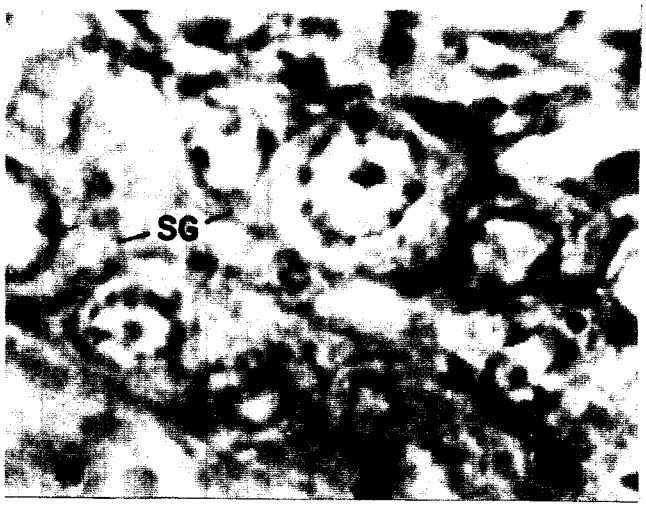
Fig. 6. Electron micrograph of the primary spermatocytes of Mugil cephalus showing the cytoplasmic extensions of the Sertoli cell (SR) forming the cyst wall, NE= Nuclear envelope; NL= Nucleolus; NU= Nucleus; SC= Spermatocyte; Arrow shows cell membrane.

PLATE XXI



20 µm

1

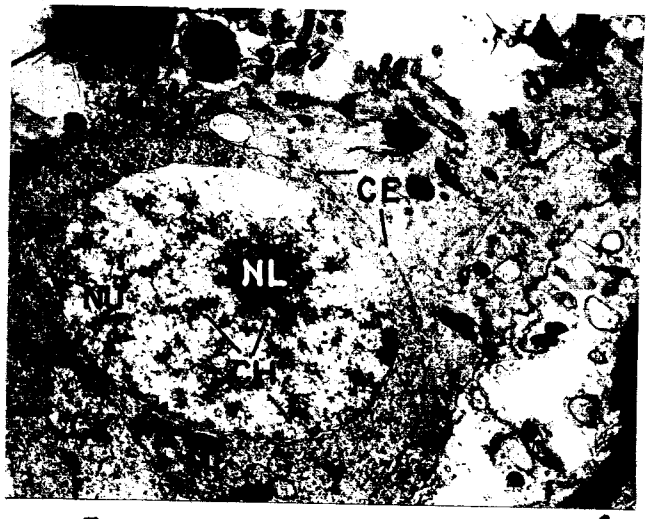


10 µm

2

5 µm

3

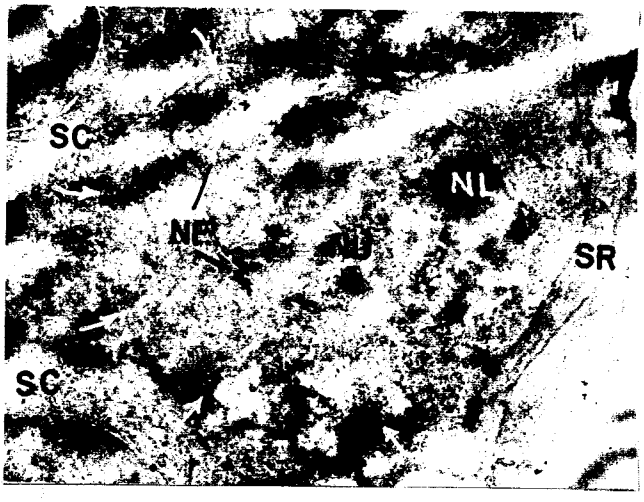


5 µm

4

3 µm

5



3 µm

6

or Sertoli cells, the cystoplasmic processes of which almost surround them (Plate XXI, Figs. 3 and 4).

The spermatogonia are ovoid cells with regular membrane and measure about 11.8 to 13.6 /um in length and 10.5 to 13.3 /um in width. The nucleus is large and measures about 8.9 to 9.3 /um in length and 6.3 to 6.4 /um in width. The nucleus has a distinct nucleolus measuring 1.5 to 2 /um in diameter. The nuclear membrane is smooth. The chromatin matter is granular and dispersed, but small clumps are found associated with the nucleolus. The cytoplasm is more electron dense than the nucleus. A few ribosomes and a number of mitochondria are prominantly visible in the cytoplasm. The ratio of nucleus to cytoplasm is 0.367.

Primary spermatocyte

Primary spermatocytes develop by the mitotic divisions of the spermatogonia. They are present in the form of cysts along the inner margin of the seminiferous lobule. Usually a single spermatogonium gives rise to a cyst of primary spermatocytes. The cyst wall is made up of the cytoplasmic processes of the sertoli cells (that initially surrounded the spermatogonia) (Plate XXI, Fig. 6). The primary spermatocytes are much smaller than the spermatogonia, ovoid, and measure about 7.1 to 9.2 /um

in length and 7.0 to 7.3 /um in width. The nucleus is also ovoid and had a length of 4.9 to 6.1 /um and a width of 3.8 to 4.3 /um (Plate, XXI, Fig. 5). The nucleoli are not distinct in all the cells. The chromatin matter is evenly distributed. The nucleus and cytoplasm is evenly electron dense. In some cysts the chromatin is seen in the condensed form. The mitochondria are very much reduced in size and number. The nucleus/cytoplasm ratio (0.435) is found to be higher than that of spermatogonism because of the reduction in the cytoplasmic volume.

Secondary spermatocyte

Secondary spermatocytes arise by the first meiotic division of the primary spermatocytes. They are present in cysts as synchronously dividing cells (Plate XXII, Fig. 1). The nuclear divisions are followed by incomplete cytoplasmic divisions thereby leaving cytoplasmic connections between the adjacent cells. The nucleolus is no longer visible. The chromatin matter is granular and distributed in the form of irregular strands within the nucleus. There is only a little cytoplasm in the cell. The cells measure about 6.4 /um in diameter and the nuclei about 3.3 /um. The ratio of the nucleus to the cytoplasm varies between 0.48 and 0.60. The secondary spermatocytes undergo the second meiotic division and give rise to spermatids.

PLATE XXII

Fig. 1. Electron micrograph of a cyst of secondary spermatocytes of Mugil cephalus. CB= Cytoplasmic bridge; NU; Nucleus; Arrow shows cytoplasmic membrane.

Fig. 2. Electron micrograph showing a cyst of electron dense early spermatids of Mugil cephalus. CB= Cytoplasmic bridge; DC= Distal centriole; IL= Intercellular lumen; PC= Proximal centriole; SD= Spermatid; Arrow shows beginning of flagellar formation.

Fig. 3. Electron micrograph of spermatids of Mugil cephalus (enlarged). CB= Cytoplasmic bridge; CH= Chromatin; DC= Distal centriole; GB= Golgi body; IL= Intercellular lumen; PC= Proximal centriole.

Fig. 4. Electron micrograph of spermatids of Mugil cephalus with condensed chromatin matter and clear nucleoplasm. CH= Chromatin; FL= Flagellum; NP= Nucleoplasm.

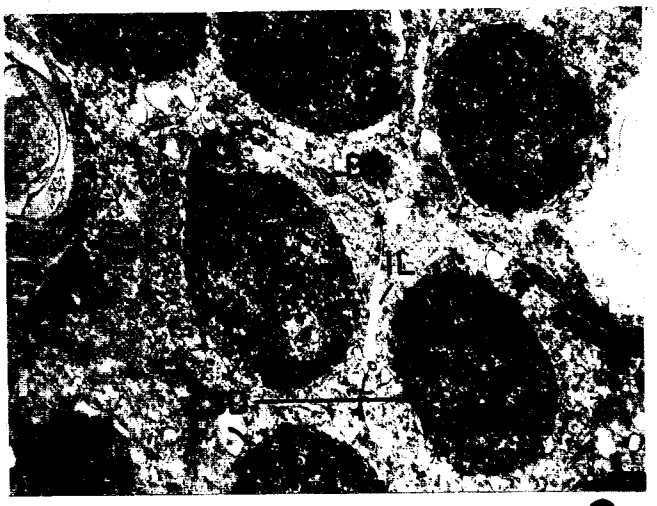
Fig. 5. Electron micrograph of late spermatids of Mugil cephalus. Note the tangentially placed flagellum and Kidney shaped chromatin mass. CH= Chromatin; FL= Flagellum; MI= Mitochondria.

Fig. 6. Photomicrograph of a spermatozoon of Mugil cephalus. SH= Sperm head; ST= Sperm tail. Aceto orcein.

PLATE XXII

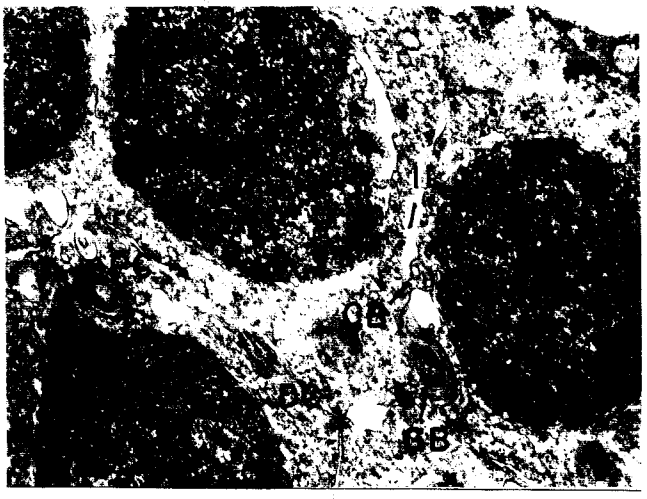
3 µm

1



2 µm

2



1 µm

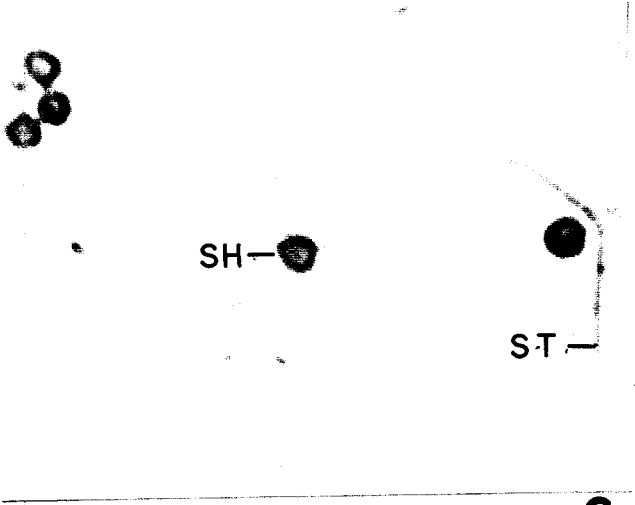
3

2 µm

4

2 µm

5



10 µm

6

Spermiogenesis

The process by which spermatids differentiate themselves into spermatozoa is known as spermiogenesis. It involves only a cellular reorganisation and no cell division. Thus the number of spermatozoa formed at the end of spermiogenesis is the same as the total number of spermatids present.

Spermatid

Spermatids are formed by the last cell division in the sequence of spermatogenesis. From the moment they are formed they are constantly undergoing cellular transformation. The early stage spermatids are characterised by the beginning of the condensation of chromatin matter and the appearance of intercellular lumen within the cyst. The appearance of the lumen is due to the shrinkage of the cells. The spermatid at this stage has a length of 4.35 ± 0.18 /um and a width of 3.33 ± 0.23 /um and the nucleus measures about 2.8 to 3.1 /um in length and 2.0 to 2.5 /um in width(Plate XXII, Figs. 2 and 3). The proximal and the distal centrioles are distinctly seen in the cytoplasm. As the development proceeds the cells undergo further shrinkage and the chromatin material further condenses and appears as a highly electron dense region within the nucleus. The nucleus at this stage has a diameter of about 1.83 /um (Plate XXII, Fig. 4). The

condensed chromatin gradually draws towards one corner of the nucleus leaving a clear region of nucleoplasm. The distal centriole begins to migrate towards the nucleus initiating the formation of the flagellum. The intercellular lumen enlarges and the cytoplasmic connections are reduced to narrow cellular bridges. The chromatin matter undergoes further condensation and becomes highly electron dense. An invagination of the nuclear mass occurs close to the region of the proximal centriole. The distal centriole by now has already migrated from the periphery of the cell forming the flagellum. The flagellum initially lies tangential to the elongated axis of the nuclear mass (Plate XXII, Fig. 5). The invagination of the nucleus becomes more prominant and the nuclear mass assumes a kidney shape. The kidney shaped nucleus rotates through 90 degrees so as to accommodate the distal centriole just beneath the nuclear notch. The nuclear mass thus becomes almost perpendicular to the flagellum which is now attached to the distal centriole. The mitochondria are observed close to the flagellum in the proximity of the nucleus. The spermatids are still found to be interconnected by thin cytoplasmic bridges and measure about 3.0 ± 0.3 /um in diameter with a nuclear cytoplasmic ratio of 0.73.

Spermatozoa

Spermatozoa are the fully developed gametes lying freely in the lumen of the seminiferous lobule.

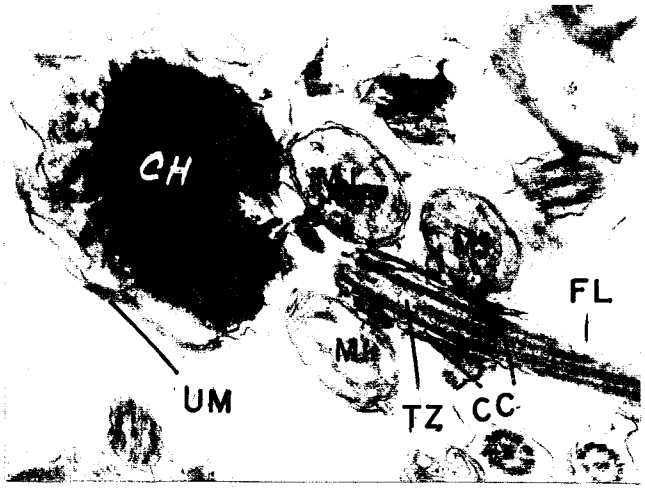
They are not interconnected by cytoplasmic bridges. Each spermatozoon is essentially made up three regions - the head, the neck, and the flagellum (Plate XXII, Fig. 6 and Plate XXIII, Fig. 1).

Head: It is made up of the condensed chromatin matter in the form of dense granules. It is kidney shaped and surrounded by two undulating membranes - an inner nuclear membrane surrounding the chromatin matter and an outer cell membrane surrounding the nucleus and the mitochondria. The outer membrane is continuous with the flagellar cytoplasmic membrane. The chromatin matter alone measures about 1.16 to 1.25 /um in length and 0.90 to 1.0 /um in width. The entire head measures about 1.53 to 1.8 /um in diameter. Lodged in the nuclear pit is the proximal centriole.

Neck: This region is made up of four mitochondria surrounding the flagellum at the region of its union with the head (Plate XXIII, Fig. 2). The mitochondria have a diameter of 0.48 to 0.83 /um. They are arranged in the form of a ring around the cytoplasmic canal of the flagellum, at the point where the flagellum meets the distal centriole.

Flagellum: The flagellum is lined by the plasma membrane throughout its length. At the region of the neck,

PLATE XXIII

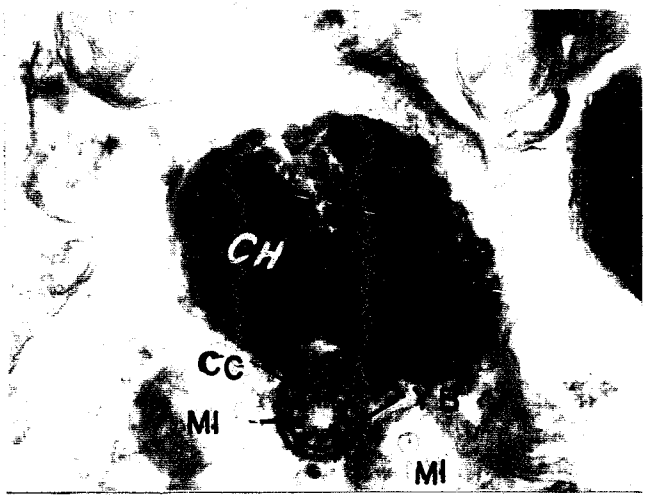


1 µm

1

0·5 µm

2



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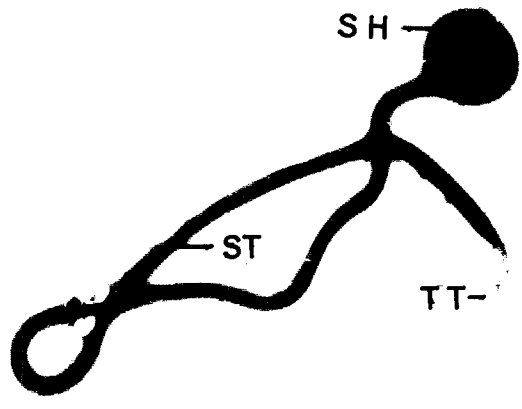


5 µm

4

PLATE XXIII

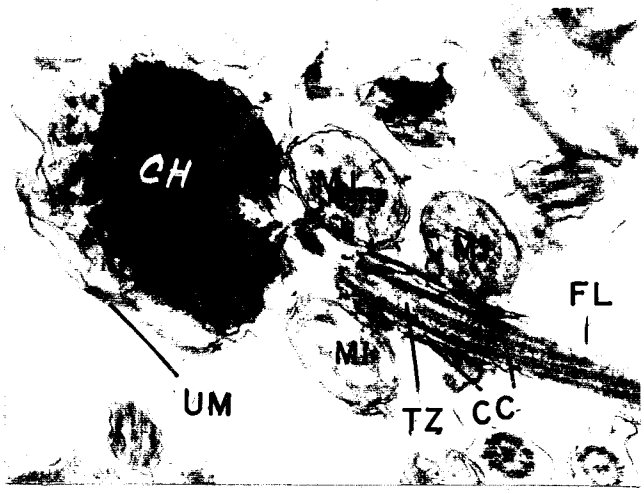
Fig. 1. Electron micrograph of the longitudinal section of a spermatozoon of Mugil cephalus. CC= Cytoplasmic canal; CH= Chromatin matter; FL= Flagellum; MI= Mitochondria; TZ= Transition zone; UM= Undulating membrane.

Fig. 2. Electron micrograph of the transverse section of the spermatozoon in the region of the midpiece showing the four mitochondria. CC= Cytoplasmic canal; FL= Flagellum; MI= Mitochondria; UM= Undulating membrane.

Fig. 3. Electron micrograph of the transverse section of the spermatozoon through the transition region of the tail showing the nine peripheral doublets and no central ones. CC= Cytoplasm canal; CH= Chromatin; MI= Mitochondria; YB= 'Y' shaped bridge; Arrow indicates the peripheral doublets.

Fig. 4. Transmission electron micrograph of a complete sperm of Mugil cephalus, showing the tapering of the tip of the sperm tail (ST). SH= Sperm head; TT= Tapering tip.

PLATE XXIII

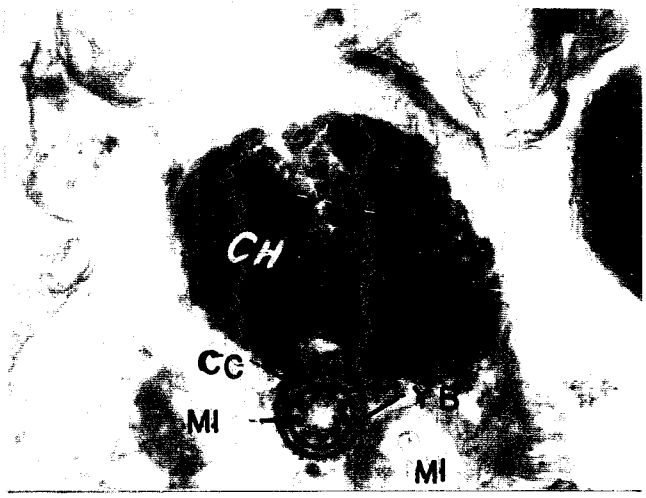


1 µm

1

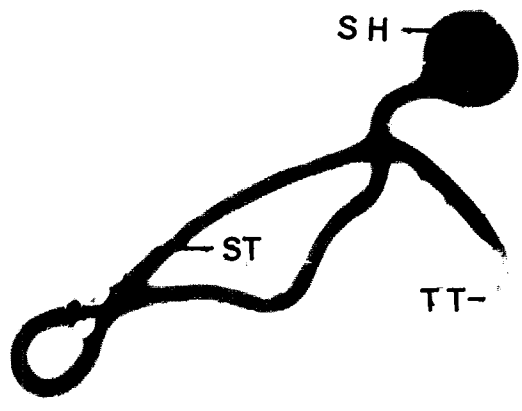
0·5 µm

2



0·5 µm

3



5 µm

4

the plasma membrane folds back on itself so as to include the mitochondria. Thus at this region, the flagellum appears to lie within a canal (cytoplasmic canal) of about 0.4 /um in diameter. The flagellum in cross section reveals a central axoneme with the typical fibrillar organisation of 9 doublets in the periphery and two singlets in the centre. The flagellum has a diameter of 0.33 to 0.45 /um while the axoneme has a diameter of 0.22 /um approximately. At the region where the flagellum is in contact with the distal centriole, the flagellar cross section shows only the nine peripheral doublets and no central component (Plate XXIII, Fig. 3). This region is called the transition zone. The doublets are connected to the cytoplasmic membrane around it by 'Y' shaped bridges. The distal end of the flagellum is characteristic in that it becomes suddenly constricted and narrow (Plate XXIII, Fig. 4). This region has a length of about 1.36 /um and a diameter of 0.16 to 0.227 /um. The length of the flagellum ranges between 30.45 /um and 36.33 /um.

SPERMATOGENESIS IN LIZA PARSIA

The structural morphologies of the different cell types and the general pattern of transformation of the

primordial germ cell into the mature spermatozoa, through the different stages of development in L. parsia are similar to that of M. cephalus. The salient features of each of these cell types are as follows.

Primordial germ cell

Primordial germ cells are large ovoid cells with highly electron dense cytoplasm. The nuclear and the cytoplasmic membranes are irregular (Plate XXIV, Fig. 1). The cell measures about 17 to 19 /um in length and 11.3 to 13 /um in width, while the corresponding measurement of the nucleus is about 11 to 11.2 and 6.8 to 7.0 /um. The cytoplasm contains a large number of organelles of which the endoplasmic reticulum and the mitochondria are clearly seen. Some electron dense substance is also seen in the cytoplasm. The mitochondria have a diameter of 5.6 microns. The ratio of nucleus to cytoplasm is 0.36.

Spermatogonia

Spermatogonia are slightly ovoid cells with smooth, regular and distinct nuclear and cytoplasmic membranes (Plate XXIV, Fig. 2). The nucleus is slightly eccentric. The cytoplasm is not as dense as that in the primordial germ cell. It has fewer organelles. Light microscopic observations reveal that the cytoplasm around the nucleus

PLATE XXIV

Fig. 1. Electron micrograph showing the ultrastructure of the primordial germ cell of Liza parsia. CE= Cell membrane; CY= Cytoplasm; DS= Electron dense substance; MI= Mitochondria; SR= Sertoli cells. Arrow indicates nuclear envelope.

Fig. 2. Electron micrograph of the spermatogonium of Liza parsia. DS= Electron dense material; MI= Mitochondria; NU= Nucleus; SG= Spermatogonium; Arrow indicates the nuclear envelope.

Fig. 3. Transverse section of the testis of Liza parsia showing spermatocyte (SC), sertoli cell (SR) and spermatogonia (SG). Note the dense cytoplasm around the nucleus of the spermatogonium; CY= Cytoplasm; NU= Nucleus.

Fig. 4. Electron micrograph showing the ultrastructure of the primary spermatocyte of Liza parsia. CE= Cell membrane; CY= Cytoplasm; NU= Nucleus; Arrow indicates the nuclear envelope.

Fig. 5. Electron micrograph of a cyst secondary spermatocytes of Liza parsia. CB= Cytoplasmic bridge; CY= Cytoplasm; NU= Nucleus; SC= Spermatocytes.

Fig. 6. Electron micrograph of a cluster of early spermatids of Liza parsia. CB= Cytoplasmic bridge; SD= Spermatid.

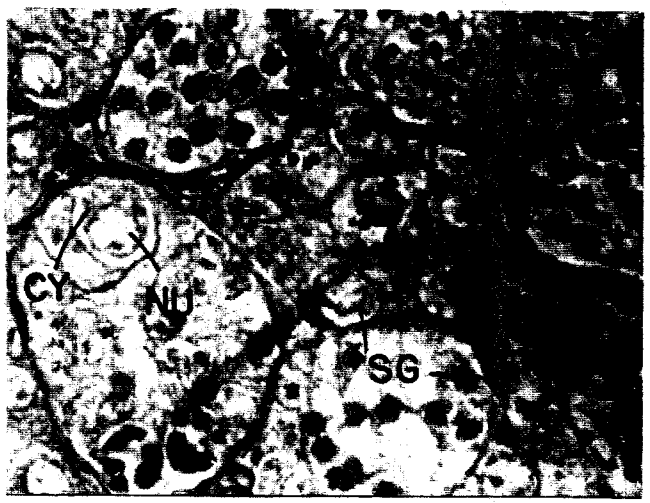
PLATE XXIV

5 µm

1

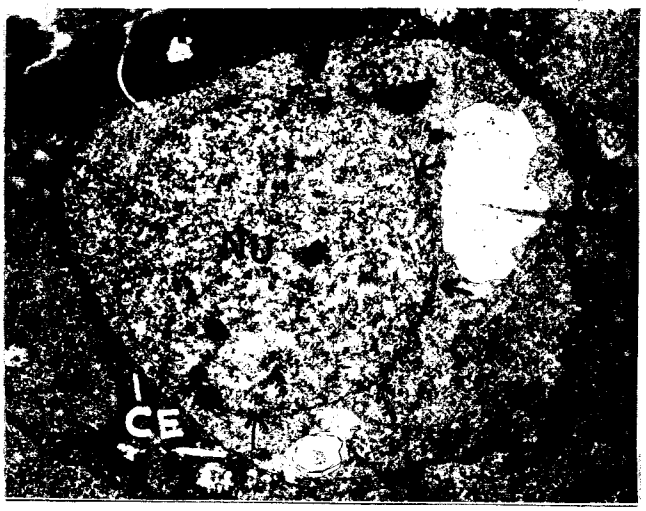
5 µm

2



20 µm

3

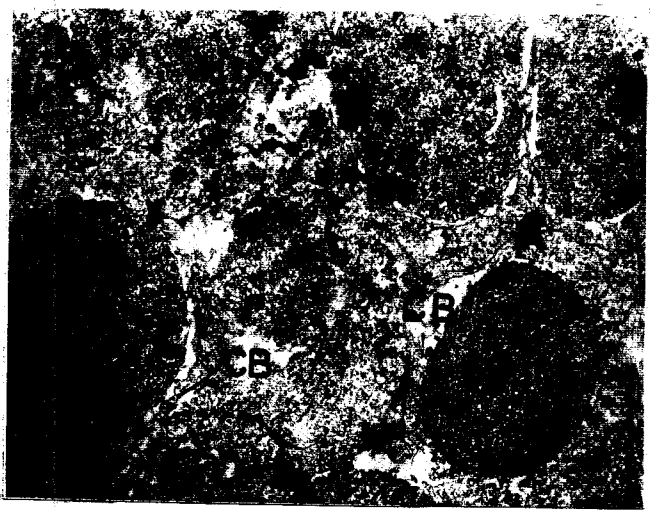


5 µm

4

5 µm

5



5 µm

6

is more dense than that in the peripheral region of the cell, adjacent to the membrane (Plate XXIV, Fig. 3). The cell measures about 16 to 17.5 /um in length and 11 to 14 /um in width, whereas the nucleus is about 9.8 to 10 /um long and 7.0 to 7.3 /um wide. The ratio of the nucleus to the cytoplasm is 0.442.

Primary spermatocyte

Primary spermatocytes are found clustered together in cysts. There are very few cytoplasmic organelles and the nucleus is more electron dense than the cytoplasm (Plate XXIV, Fig. 4). The chromatin matter is present as evenly distributed granules, throughout the nucleoplasm. The cell is about 12.4 to 14.8 /um in length and 10 to 12 /um in width. The corresponding measurements of the nucleus is 7.0 to 7.3 /um and 5.1 to 6.8 /um respectively. Ratio of nucleus to cytoplasm is 0.5.

Secondary spermatocyte

The primary spermatocyte by the first meiotic division (reduction division) gives rise to a group of synchronously dividing cells - the secondary spermatocytes (Plate XXIV, Fig. 5). These cells show intercellular cytoplasmic bridges which arise by the incomplete divisions of the cell. The nuclear material is dense. The cell measures about 6.6 to 8.07 /um in length and 5.7 to 6.1 /um

in width. The corresponding size of the nucleus is about 4.4 to 6.6 /um and 3.3 to 4.3 /um respectively. The ratio of nucleus to the cytoplasm is 0.512.

Spermiogenesis

Spermatid

As in M. cephalus, a series of functional morphological changes associated with the transformation of the spermatids to spermatozoa occur in this stage. These changes begin with the shrinkage of the cytoplasm, condensation of the chromatin material and the enlargement of the intercellular spaces. The nuclear diameter at the beginning of these changes is about 2.71 $\pm$ 0.7 /um and that of the cell about 4.7 $\pm$ 0.33 /um (Plate XXIV, Fig. 6). The distal centriole migrates forming the tail. The nucleus then assumes the kidney shape and measures about 2.0 $\pm$ 0.21 /um in length and 1.65 $\pm$ 0.13 /um in width (Plate XXV, Fig. 1). The nuclear notch is 0.86 /um in depth. Initially the flagellum is tangential to the nucleus (Plate XXV, Fig. 2). The rotation of the nucleus through 90 degrees takes place here also so as to accommodate the flagellum in the nuclear notch. The nucleus and the flagellum thus become perpendicular to to each other and the nuclear cytoplasmic ratio is about 0.748.

PLATE XXV

Fig. 1. Electron micrograph of spermatids of Liza parsia showing chromatin condensation and the Kidney shape formation. FL= Flagellum; SD= Spermatid; SZ= Spermatozoa.

Fig. 2. Electron micrograph of the longitudinal section of a late spermatid of Liza parsia with tangentially placed flagellum. CC= Cytoplasmic membrane; CH= Chromatin material; FL= Flagellum.

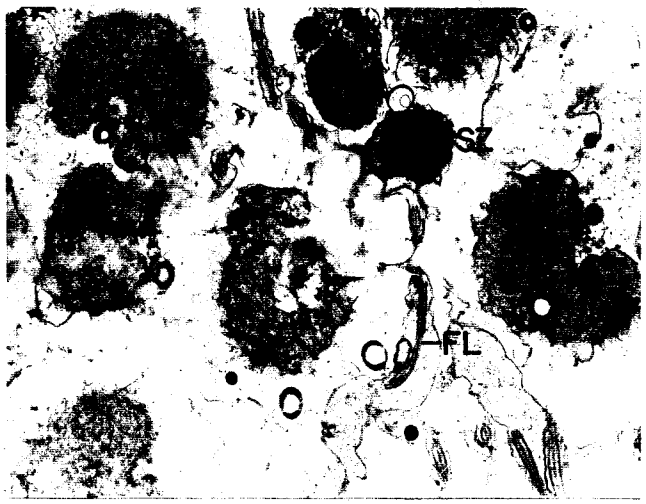
Fig. 3. Photomicrograph of a single sperm of Liza parsia. SH= Sperm head; ST= Sperm tail. Acetoorcein.

Fig. 4. Electron micrograph of a section passing through spermatozoa of Liza parsia. CH= Chromatin material; DC= Distal centriole; FL= Flagellum; MI= Mitochondria; PC= Proximal centriole.

Fig. 5. Electron micrograph of the longitudinal section of the flagellum. CC= Cytoplasmic canal; MI= Mitochondria; TZ= Transition zone.

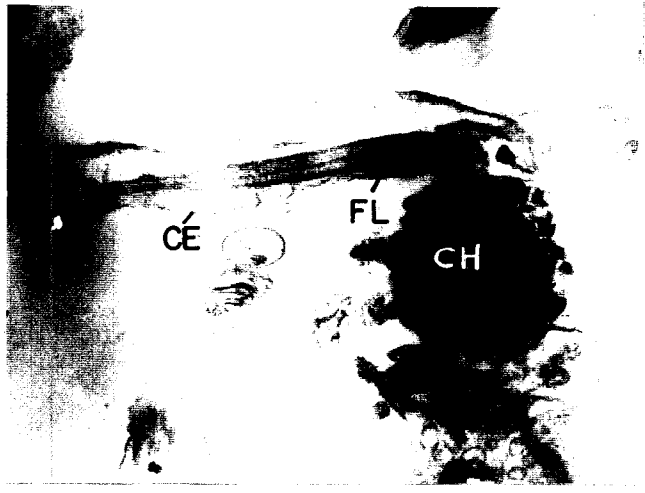
Fig. 6. Transmission electron micrograph of a complete sperm of Liza parsia, showing the tapering of the sperm tail. SH= Sperm head; ST= Sperm tail; TT= Tapering tip.

PLATE XXV



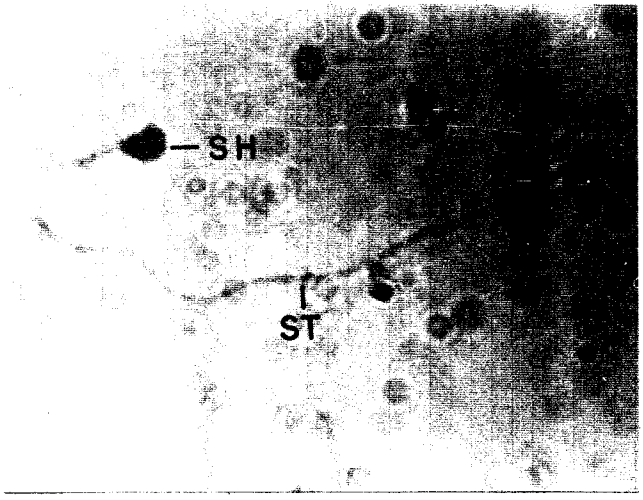
2 µm

1



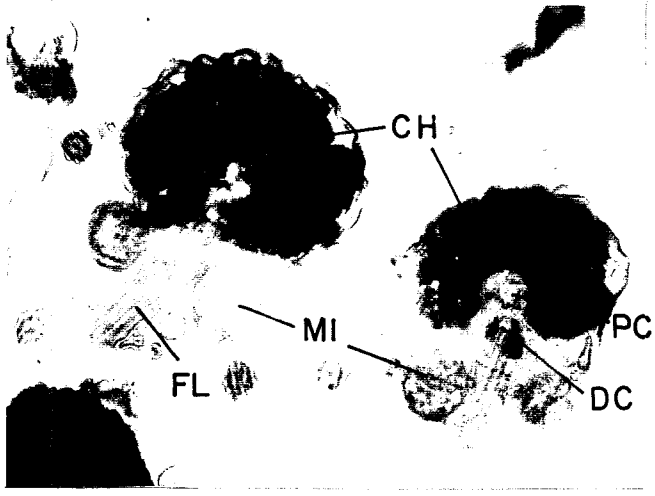
1 µm

2



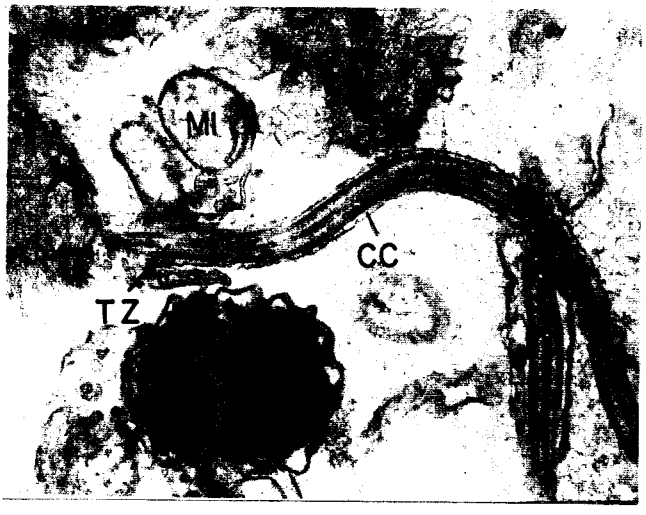
10 µm

3



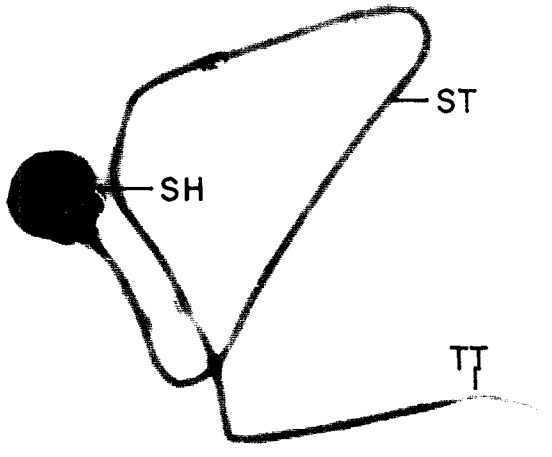
1 µm

4



1 µm

5



5 µm

6

Spermatozoa

The mature sperm of L. parsia is also composed of head, mid piece and tail or flagellum (Plate XXV, Fig. 3). The chromatin matter, highly condensed and granular and surrounded by a membranous envelope, forms the head of the spermatozoa (Plate XXV, Fig. 4). These cells are totally devoid of cytoplasmic bridges. The kidney shaped head has a diameter of 1.83 to 1.87 /um. The nucleus has a length of 1.58 to 1.66 /um and a width of 0.83 to 0.91 /um. Four mitochondria of about 0.5 to 0.65 /um in diameter form a ring at the region where the flagellum unites with the head. The flagellum is about 0.25 to 0.32 /um in diameter and has a typical 9+2 organisation. It is surrounded by a cytoplasmic canal which has a diameter of 0.38 /um. The proximal centriole is distinctly seen lodged in the nuclear pit of the kidney shaped head. At the transition zone of the flagellum, the middle tubules are found to be absent (Plate XXV, Fig. 5). The flagellum has a length of about 36.36 to 41.25 /um. The terminal extremity of the flagellum is found to be gradually tapering for the last 2.5 /um reaching a diameter of about 0.16 to 0.18 /um (Plate XXV, Fig. 6).

DISCUSSION

The term "germ cell" was used by Turner (1919) to identify the first formed primordial cells in the testes of the perch P. flavescens. The subsequent cell types were called spermatogonium, spermatocyte, spermatid and spermatozoa based on their size, behaviour and cytoplasmic and nuclear characteristics. However, different views were expressed on the origin and differentiation of the germ cell to subsequent cell types and on the terminology used to designate the different cell types. Such early workers as Hoffman (1886) and Bohi (1904) thought that the germ cells originated from the germinal epithelium lining the tubules, while others believed that they had an extra gonadal origin in the embryonic stage (Eigenmann, 1891; Beard, 1900; Allen, 1911; Bachman, 1914; Dodds, 1910; Okelberg, 1921; Hann, 1927; Stromstein, 1931 and Robertson, 1953). Still others (Essenberg, 1923; Foley, 1927, Butcher, 1929; Odum, 1936 and Guerbilsky, 1939) were of the view that the sperm and the ova developed not only from the primordial germ cells but also from the somatic cells. Johnston (1951), working on Micropterus salmoides salmoides claimed that the primary germ cells were differentiated from the blood cells. Supporting this view, Jordan (1917) and

Risely (1933) showed the similarities in the morphological structure between the germ cells and blood cells. Essenberg (1923) working on swordtail concluded that the duct epithelial cells gave rise to spermatocytes and spermatids. Wolf (1931) however disagreed with this view and reported that in the swordtail and platy fish, sperms were derived from the primordial germ cells and that the duct epithelium did not form the germ cells. Turner (1919) working on perch identified germ cells of various shapes and sizes and assumed them to be in their migratory phase. He opined that the germ cells after assuming their position in the seminiferous lobules, underwent proliferation and growth before differentiating to spermatogonia. Turner's studies supported the theory/extragonadal origin of germ/ of cells and also suggested that migration of germ cells continued to take place even after the gonad attained maturity.

The earliest work in Mugilidae, on this aspect was by Stenger (1959). As his studies commenced only from the 20 mm size fry, he was not able to trace the origin of germ cells. Nevertheless, he ruled out the possibility of the origin of the germ cells in M. cephalus from either stromal cells or blood cells. Further, his studies clearly indicated that the duct epithelial cells, played no part in the formation of the germ cells. It is now generally accepted that the primordial germ cells

in teleosts, as in other vertebrates, originate extragonadally and migrate to the gonadal region (Nagahama 1983).

Following Turner (1919), Stenger used the terms such as spermatogonia, spermatocytes and spermatids, to describe the different cell types in his classical work on the spermatogenesis of M. cephalus. But he did not identify any amoeboid germ cells as observed by Turner (1919), yet mentioned a period of cell multiplication, followed by shrinkage in size, during the transformation of the germ cells into a spermatogonium. Michibata (1975) differentiated three types of spermatogonia in the testes of medak, "spermatogonia A" (3-4 µm in diameter), "spermatogonia differentiated" (5 - 10 µm in diameter) and "spermatogonia B" (5 µm in diameter). Kristoffersson and Pekkarinen (1975) used the term "primary germ cell" for the gonadal cells which formed the predecessors of spermatogonia, while Remacle et al. (1977) recognized two types of spermatogonia namely "primary spermatogonia" and "secondary spermatogonia".

Billard (1979) working on salmonids, used the term "stem spermatogonia" to describe the cells which originated from the embryonic germ cells, which were called "gonocytes". According to him, the 'stem spermatogonia' were present permanently in the gonad and were also called 'type A spermatogonia' following the terminology employed in mammals. These cells were believed to give rise to a second type of

spermatogonia, 'type B spermatogonia', which further differentiate to cysts of spermatocytes.

Brusle and Brusle (1978a, 1978b) studying the spermatogenesis in L. auratus and Brusle (1980) describing the ultrastructure of the early germ cells of M. cephalus designated the first formed germ cells as "primordial germ cells". These cells were considered to be bipotential cells, being present both in males and females, during the embryonic development of the gonad. These authors however, discussed only one type of spermatogonia. The subsequent stages of spermatogenesis described by them were the primary spermatocyte, secondary spermatocyte, spermatid and spermatozoon. Strangely they did not mention about the division of primordial germ cell to form spermatogonia. Hurk (personal communication) agrees with Billard's classification of spermatogonia into 'type A' and 'type B'. He considers the presence of a distinct nucleolus within the nucleus as an indication of the spermatogonial stage and hence in this respect, he disagrees with Brusle and Brusle (1978a, 1978b) who observed the presence of a nucleolus even in the primary spermatocytes.

In the present study, six spermatogenic cell types are differentiated in the testes of both M. cephalus and L. parsia. They are, the primordial germ cell, the spermatogonium, primary spermatocyte, secondary spermatocyte,

spermatid and spermatozoon or sperm. The cellular structure of these cells, was found to be similar to that described by Brusle and Brusle (1978a, 1978b) and Brusle (1980, 1981c).

The salient features of the various cell types of these two species along with those of L. aurata are given in tables 5 and 6. In all the three species, the primordial germ cells (PGC) are characterised by their electron dense cytoplasm, low nuclear cytoplasmic (N/C) ratio and irregular shape, although the PGC is relatively larger in L. parsia than those in the other two species. As compared to the PGC the spermatogonia are fairly regular cells, with less electron dense cytoplasm and large nucleus in all the three species discussed. In L. parsia certain spermatogonia, were found to possess a densely staining region of cytoplasm around the nucleus. This situation was not observed in M. cephalus. Turner (1919) however observed a similar condition in perch (P. flavescens) and suggested that it could be an indication of the synthetic phase of the cell. In most vertebrates, especially mammals the synthetic and the growth phase of the germinal cells is prominantly seen in the primary spermatocyte stage. However in teleosts, there is a gradual decrease in the size of the cell from the spermatogonium to the spermatozoon, indicating that the growth phase of the cell almost completes at the spermatogonial stage itself.

TABLE - 5. Early germ cell types of *M. cephalus*, *L. parsia* and *L. auratus*

Characters	*M. cephalus* (present study)			*L. parsia* (present study)			*L. auratus* (Brusle 1978)		
	Primordial germ cell	Spermatogonia	Primary spermatocyte	Primordial germ cell	Spermatogonia	Primary spermatocyte	Primordial germ cell	Spermatogonia	Primary spermatocyte
1	2	3	4	5	6	7	8	9	10
Topography	Isolated or clusters	Isolated or in clusters & cysts	in cysts	Isolated or clusters	Isolated or in clusters and cysts	in cysts	Isolated or clusters	in nests or cysts	in cysts
-Cytoplasmic bridges	-	-	-	-	-	-	-	-	++
MORPHOLOGY									
- Shape	Irregular	Ovoid	Ovoid	Irregular	Slightly ovoid	ovoid	ovoid with irregulat outline	ovoid with slightly irregular outline	ovoid with regular outline
- Size Length	10-11.5 µm	11.8-13.6 µm	7.1-9.2 µm	17.0-19.0 µm	16.0-17.5µm	12.4-14.8 µm	11.7-15.3µm	10.9-17.5 µm	9.5-13.5 µm
Width	8.5-9.0 µm	10.5-13.3 µm	7.0-7.3 µm	11.3-13.0 µm	11.0-14.0µm	10.0-12.0 µm	7.8-11.2µm	8.0-12.5 µm	5.4- 8.6 µm

Contd.....

TABLE - 5.(Contd.)

1	2	3	4	5	6	7	8	9	10
CYTOLOGY									
Nucleus:									
- Shape	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid nearly round
Size Length	6-6.5 µm	8.9-9.3 µm	4.9-6.1 µm	11.0-11.2 µm	9.8-10 µm	7.0-7.3 µm	6.4-9.6 µm	6.4-10.2 µm	5.5-9.1 µm
Width	4-5.0 µm	6.3-6.4 µm	3.8-4.3 µm	6.8-7.0 µm	7.0-7.3 µm	5.1-6.8 µm	3.9-7.1 µm	5.2-8.8 µm	4.5-7.1 µm
Position	eccentric	eccentric	almost central	eccentric	eccentric	almost central	eccentric	eccentric	almost central
Nuclear envelope	irregular	smooth, regular	smooth regular	irregular	regular	regular	irregular	slightly irregular	regular
Chromatin	distributed	granular, dispersed and also in clumps	evenly distributed	evenly distributed	distributed	evenly distributed	finely granular dispersed	granular with clumps	granular dispersed
Cytoplasm	electron dense	less electron dense	not electron dense	electron dense	less electron dense	not electron dense	electron dense	less electron dense	not electron dense
Nuclear/ cytoplasmic ratio	0.30	0.367	0.435	0.36	0.442	0.50	0.38±0.07	0.4± 0.06	0.41± 0.08
Mitochondria	few	many	few	few	many	few	many	many	few
Electron dense substance	-	-	-	+	+	-	++	++	++

- Absent; + Present in one or two numbers ; ++ Present in more than two numbers

TABLE - 6. Morphological and cytological features of secondary spermatocytes and spermatids of Mugil cephalus, Liza parsia and L. auratus.

Characters	Mugil cephalus (present study)			Liza parsia (present study)			L. auratus (Brusle 1978)		
	Secondary spermatocyte	Early spermatid	Late spermatid	Secondary spermatocyte	Early spermatid	Late spermatid	Secondary spermatocyte	Early spermatid	Late spermatid
TOPOGRAPHY	In cysts	In cysts	In cysts	In cysts	In cysts	In cysts	In cysts	In cysts	In cysts
- Cytoplasmic bridges	+	+	+very narrow	+	+	Not visible	+	+	+very narrow
- Intercellular spaces	narrow	large	very large	very narrow	narrow	very large	narrow	narrow	very large
MORPHOLOGY -Shape	ovoid or round	ovoid or round	irregular	ovoid or round	ovoid or round	irregular	ovoid	ovoid	irregular
-Size: Length	6.4±0.56 µm	4.35 ±0.18µm	3.0±0.30 µm	6.6-8.07 µm	4.7 ±0.33 µm	4.2 ±0.24 µm	6.6±0.61 µm	5 - 6 µm	*
Width	(diameter)	3.33±0.23µm	(diameter)	5.7-6.1 µm	(diameter)	(diameter)	4.5±0.60 µm	(diameter)	*
CYTOLOGY NUCLEUS -Shape	round	ovoid	kidney shaped	ovoid	ovoid	kidney shaped	ovoid	ovoid	Kidney shaped
-Size: Length	3.3±0.25 µm	2.8-3.1 µm	1.5±0.14 µm	4.4.6.6 µm	2.71±0.7 µm	2.00±0.21 µm	*	4 - 5 µm	*
Width	(diameter)	2.0-2.5 µm	1.107±0.2µm	3.3-4.3 µm	(diameter)	1.65±0.13 µm	*	(diameter)	*
- Chromatin	Thread like	Fine granular and electron dense	densely granular and electron dense	granular	fine granular	densely granular and electron dense	irregular strands	irregular strands	densely granular
-N/C ratio	0.48 to 0.6	0.38	0.73	0.512	0.33	0.748	0.4	0.4	*
CENTRIOLES	Not distinct	Distal centrioles begin to migrate	Proximal near nuclear notch; distal at beginning of the flagellum	Not distinct	not distinct	Proximal near the nuclear notch; Distal at the beginning of the glafellum	*	Both centrioles lie near the cell membrane Distal shows migration	Distal at the beginning of the flagellum
FLAGELLUM	-	-	Tangential to the nucleus	-	-	Tangential to the nucleus	-	-	Initially tangential later perpendicular

- Absent; + Present; * Information not available

The spermatogonia of both M. cephalus and L. parsia are richly endowed with organelles. This observation agrees with that of Brusle and Brusle (1978b) but contrasts with the observation on other fishes such as Oryzias (Satoh and Egami, 1973), Carassius (Remacle et al., 1977) and Platypoecilus (Russo and Pisano, 1973) in which spermatogonia are characterised by the lack of such organelles.

The primary spermatocytes of M. cephalus were much smaller than that of L. parsia. It is interesting to note that, the cytoplasmic bridges resulting from incomplete cytoplasmic divisions of adjacent cells, observed in the primary spermatocytes of L. auratus (Brusle and Brusle, 1978a, 1978b) were not seen in the primary spermatocytes of M. cephalus and L. parsia.

The secondary spermatocytes are much smaller cells as compared to the primary spermatocytes, with condensed chromosomes, undergoing the second meiotic division. Unlike in primary spermatocytes, the intercellular bridges between the secondary spermatocytes could be clearly seen.

Although the spermatids of M. cephalus and L. parsia are similar in structural details, the size of the cell as well as the nucleus of the latter species is relatively larger than that of the former. At this stage the cells

undergo drastic structural reorganisation, before they transform into spermatozoa. Unlike the sperms of other vertebrates, the teleost spermatozoon is devoid of an acrosome and hence the usual golgi complex involvement in acrosome formation seen in other vertebrate spermatids is absent here. The major events occurring at this stage are the condensation of chromatin matter, elongation of the nucleus and the cell, invagination of the nucleus to form the kidney-shaped head of the future spermatozoon, migration of the distal centriole, formation of the tail, migration of the mitochondria and finally the rotation of the head through 90 degrees to accommodate the tail beneath the nuclear notch. These events of spermatogenesis are similar in both M. cephalus and L. parsia and are comparable with the same of L. auratus (Brusle, 1981c).

Thin walled somatic cells (Sertoli cells) forming the outer limits of spermatogenic cysts were observed in the testes of both the species taken up for the study. The structure and function of these cells have been studied in great detail by Billard (1970b), Hurk et al. (1974b) and Grier (1975). In the Poecilidae, at the final stage of spermatogenesis, the sperm nuclei become embedded in the Sertoli cell cytoplasm while in Goddidae, the spermatid flagellum is found to have a similar association with the Sertoli cell. However in the present study no such association between the Sertoli cell and the spermatid was observed.

Mattei (1970) has defined four steps in the process of spermiogenesis. These are (i) the newly formed spermatid (ii) the migration of centrioles (iii) the rotation of the nucleus and (iv) the migration of the mitochondria. Further, based on the presence or absence of the rotation of the nucleus, he (Mattei, 1970) classified spermiogenesis into type I (with nuclear rotation) and type II (without nuclear rotation). According to this classification, both the species dealt with in the present study, belong to the type I spermiogenesis.

On the basis of structural changes, Brusle (1981c) recognises five stages in the spermiogenesis of L. auratus. Each stage being a little more advanced than the preceeding one in the formation of the spermatozoa. In the present study the sequence followed seem to be similar to that observed by Brusle (1981c).

The important structural characteristics of the spermatozoa in M. cephalus and L. parsia as revealed from the present study are compared with that of L. auratus and L. dumerili in the table 7. Though the general formation of the head, middle piece and flagellum in the two species chosen for the study are similar, it is observed that in the over all size, the spermatozoa of L. parsia are larger than that of M. cephalus although it is a smaller species. Of the four species compared, L. auratus has the largest flagellum

TABLE -7. Structure of the spermatozoa of different species of Mugilidae

Characters	M. cephalus (present study)	L. parsia (present study)	L. auratus (Brusle,1981)	L. dumerili (Van der Horst and Cross, 1978)
1	2	3	4	5
Head				
Shape of the nucleus	Kidney shaped	Kidney shaped	Kidney shaped	Kidney shaped
Nuclear length	1.16 to 1.25 /um	1.58 to 1.66 /um	1.5/um(diameter)	*
Nuclear breadth	0.9 to 1.0 /um	0.83 to 0.91 /um	*	*
Diameter of complete head	1.53 to 1.8 /um	1.83 to 1.87 /um	*	2.4 /um
Centrioles	Two in number. Proximal lodged in the nuclear pit and distal at right angles to it at the beginning of the flagellum	same as in M. cephalus	same as in M. cephalus	Same as in M. cephalus
Mid piece				
Mitochondria	Four independent mitochondria 0.48 to 0.83 /um in diameter.	Four in number 0.50 to 0.65 /um in diameter.	Four in number 0.55 to 0.66 /um in diameter	Four in number 0. 6 /um in diameter

Contd .....

TABLE - 7 (Contd.)

1	2	3	4	5
Flagellum				
Length	30.45 to 36.33 /um	36.36 to 41.25 /um	> 60 /um	39 to 43 /um
diameter of flagellum	0.33 to 0.45 /um	0.25 to 0.32 /um	*	*
Cytoplasmic canal	0.4 /um (approx.)	0.38 /um (approx.)	*	*
axoneme	0.22 /um (approx.)	0.16 /um (approx.)	0.2 /um	0.23 /um
Terminal end length	1.36 /um (approx.)	2.5 /um (approx.)	*	*
diameter	0.16 to 0.227 /um	0.16 to 0.18 /um	*	*

* Information not available

and L. dumerili, the largest sperm head. In all the four species the flagellum is composed of an axoneme with the characteristic 9 + 2 arrangement surrounded by cytoplasm, that is delimited by the plasma membrane. The diameter of the axoneme is constant throughout the length of the flagellum except at the terminal end. However, the diameter of the cytoplasm containing the axoneme, varies slightly along the length of the flagellum.

In both M. cephalus and L. parsia it is observed that the diameter of the extreme end of the flagellum is much lesser than that of the rest of the flagellum. In L. parsia the tapering of the extreme end is gradual and commences at about 2.5 µm above the tip while in M. cephalus the tapering is abrupt and commences from about 1.36 µm above the tip of the tail. A similar condition was reported by Mattei et al. (1967) in Lebistes reticulatus and Grier (1975) in P. latipinna. In both the cases the reduction in the diameter towards the terminal end was reported to be due to the disappearance of the central axonemal filaments, followed by the transformation of the peripheral doublets into singlets and the gradual disappearance of the singlets. Mattei (1969) studied the termination of flagella in a number of fishes and identified two groups, one in which the central axonemal fibrils disappear first followed by changes in the doublets and the second in which the

disorganisation of the peripheral doublets begins before the disappearance of the central filaments. However in both the groups, the central filaments disappear eventually and only a few (1 - 3) peripheral filaments are retained till the tip. In the present study though the sequential changes in the termination of the flagellar tip was not taken up in detail, it is speculated that, the tapering observed in both the species must be due to the disappearance of the axial filaments followed by the reduction in number of the peripheral filaments.

Billard (1970a) classified the spermatozoa of teleost fishes into three groups on the basis of their degree of evolution. As per this classification the spermatozoa of M. cephalus and L. parsia can be included under the group I, characterised by an ovoid head and slightly tangential flagellum.

Sequence of Spermatogenesis

From the forgoing observation on the different stages of spermatogenesis, the sequence of transformation of the primordial germ cell to the mature spermatozoon, in M. cephalus and L. parsia could be summarised as follows.

The primordial germ cells make their appearance in the form of sparsely distributed darkly staining cells in

the connective tissue stroma of the immature gonad. As the maturation process progresses, these cells multiply and organise themselves into distinct lobules with an intralobular space, lined by a single layer of germ cells. Each lobule is seperated from the adjacent ones by the interlobular somatic tissue. Each primordial germ cell after a period of proliferation transforms into a spermatogonium, which can be distinguished by its smooth cell membrane, ovoid shape, regular nuclear membrane and a comparatively less electron dense nucleoplasm. The spermatogonia undergo a period of growth, thus becoming the largest germ cells in the gonad.

After the growth phase, the cell multiplies and gives rise to a group of synchronously dividing cells held together in the form of a cyst by the cytoplasmic processes of the Sertoli cells. As the proliferation of the cells in the cysts occur, the lumen of the lobule increases. All the spermatogonia do not develop into cysts. Some remain in the dormant condition. Each primary spermatocyte, in the cyst, undergoes the first meiotic division (reduction division) and gives rise to two secondary spermatocytes. Each secondary spermatocyte undergoes the second meiotic division and gives rise to two spermatids. The spermatids undergo cellular reorganisation coupled with the formation of the tail and transform

into the spermatozoa. The spermatozoa when they are initially formed assume an umbrella like arrangement with their heads facing the periphery of the cyst and their tails facing the centre.

The cells in the different cysts are initially at different stages of maturity but as the fish approaches the spawning period, most of the cysts are filled with sperms.

How spermatogonial renewal takes place, in the teleost testes, still remains a controversy, although a number of studies on the development and ultrastructure of spermatogonia have been made.

The majority of the investigators have concluded that the spermatogonia arise from germ cells (stem cells) which have been dormant in the peripheral portions of the testis (Nagahama 1983). The presence of the dormant cells in the advanced stages of spermatogenesis in L. parsia and M. cephalus support the above statement.

The duration of the spermatogenesis from the spermatocyte to the spermatozoa has been worked out in some fishes using autoradiographic studies. Egami and Hydo-Taguchi (1967) were able to find out that in Medka, the time interval for spermatogenesis from leptotene spermatocyte to spermatozoon is twelve days at 25°C and twenty days at

15°C. In guppy, Billard (1968) found that 14.5 days are required for completing the development from early leptotene to the spermatozoon at 25°C. DeFelice and Rasch (1969) observed that at least twenty one days are required for completing the same stages of development in the case of Poecilia shenops. In India similar work has been done by Ghosal et al. (1981) in Rachophorus maculatus and by Mohan Sinha et al. (1979) in Colisa fasciata. It was found that in Colisa fasciata, the leptotene lasts for less than 0.5 days, zygotene for an even shorter period and pachytene for 1.86± 0.13 days. The transition from diplotene to anaphase II requires about a day. The spermatogenesis is completed within 6.43 days.

Although autoradiographic studies could not be carried out during the present investigation, an attempt is made to determine the duration of spermatogenesis in M. cephalus specimens sampled from the natural population at the barmouth region of the Cochin backwaters. It is seen that the development from the spermatocyte, which is predominantly seen in the third stage testes collected during the months of April-May and October - November, to the motile sperm, which is found abundantly in the fifth stage testes and collected during the months of May - June and November - December, occurs within a period of two months. The specimens during the other periods have predominantly spermatogonia and a few spermatocytes.

As in other teleosts, the process of spermatogenesis in mullets is governed by hormonal control (Eckstein and Fylath 1968, Eckstein 1975) which in turn is governed by the environmental changes. Although the presence of Leydig cells in the interlobular somatic tissue was observed, a quantitative assessment of the hormones with respect to the spermatogenic stages was not made as it was not envisaged during the present work due to paucity of time. However, since information on this aspect is essential for the better understanding of the process, it is hoped that studies on this aspect will be persued by future workers with the aid of radioimmuno assays.

CHAPTER VII

BIOCHEMICAL CHANGES DURING SPERMATOGENESIS

INTRODUCTION

The reproduction in teleosts is a dynamic process mediated by several endogenous and exogenous factors which influence the development and formation of gametes, their maturation and subsequent release. Among the endogenous factors, mobilization of major nutrients and other biochemical constituents for the gamete formation and maturation are important. Although extensive work is available on the general biochemical constitution of several species of fishes, studies on biochemical changes occuring during maturation of gonads are rather scanty. This is particularly so in the case of males of the species.

Some of the earlier noteworthy works in this field were by Lovern (1934) in Salmo salar; Petrenko and Karasikova (1958) in Clupea sprattus; Krishnamoorthy (1958) in Labeo fimbriatus, Mystus seenghala and Boleophthalmus boddaerti; Braekkan and Boge (1962), Gjessing (1963) Mengi (1965) and Jangaard et al. (1967) in Gadus morhua; Idler and Bitners (1958, 1959 and 1960) in Salmo salar; Korzhenko (1966) in Oncorhyncnus keta

and the reviews by Love (1970), Iles (1965, 1974 and 1984) and Shulman (1974). These works have shown that the reproduction in fishes is an energy demanding activity and brings about drastic changes in the biochemical composition of the fish.

Investigations on the biochemical composition of the Indian fishes have been mainly restricted to the fresh water species, the widely investigated fishes being Heteropneustus fossilis, Clarius batrachus and Channa punctatus. Biochemical variations in relation to oogenesis have been worked out by Venugopalan (1962) in Ophiocephalus striatus. Similar studies on the biochemical changes occuring during different stages of maturity in both the sexes of Cyprinus carpio were carried out by Masurekar and Pai (1979), who observed a gradual depletion in the protein and fat content of the muscle tissue during maturation with values touching the minimum in the spent condition.

The biochemical composition of the grey mullets such as M. cephalus, L. dumerili, L. richardsoni and L. tricuspidens were studied by Marias and Erasmus (1977). Perera and De Silva (1978) determined the body composition of the young ones of M. cephalus. Recently Mukundan et al. (1981) reported the nutritive value of mullet along with other commercial fishes and shellfishes of the Cochin

region. These investigators , however, considered neither the maturity stage nor the sex of the material used for the study.

In the recent years, studies on the biochemistry of the different body constituents during gametogenesis have received considerable attention in view of their role in the process of gamete formation and maturation. As information on this aspect in the marine fishes of India is scarce and since such information on the grey mullets of the country is not available, the present study on the variations of five major parameters such as moisture, protein, carbohydrate, lipid and cholesterol in fresh and dry tissues of the testes, liver and muscle in M. cephalus and L. parsia at different stages of maturity, was taken up and the results discussed.

OBSERVATIONS

Biochemical composition of tissues

The data on estimated moisture, protein, carbohydrate, lipid and cholesterol content in the gonad, muscle and liver tissue of M. cephalus and L. parsia at different stages of maturity along with the corresponding standard deviations are given in tables 8 to 11 and depicted in Plates XXVI to XXXV. To obviate the influence of moisture

content on the estimation of these parameters, analysis of the various tissues was carried out on dry weight basis besides the estimation on fresh tissues.

M. cephalus

Testes

Moisture: The moisture content of the testes in different stages of maturity is given in Table 8 and Plate XXVI. A steady increase in the moisture content of the gonadal tissue was observed from the first stage (76.37%) to the fourth stage (78.03%). As the testes matured to the fifth stage, the moisture content increased by about 3% over that of the previous stage. However, in the very next stage (spent condition) it was found to decrease by about the same value.

Protein: The total protein level in the immature testes was 14.79%. As the maturation process advanced, percentage of protein was seen increasing to attain the highest value of 17.76% in the fourth stage (Plate XXVII). In the fully mature testes the highest protein level was 15.33% and it decreased further to 13.61% in the spent stage. On dry weight basis (Table 9) the total protein level was seen to increase steadily from the immature to mature stage reaching the maximum value of 82.55% in the latter stage. In the spent testes, however, it declined to 62.52% almost the same level as that of the immature stage.

TABLE 8. Biochemical composition of fresh tissues of testes, muscle and liver of M. cephalus during different stages of maturity.

Stages	Testes					Muscle					Liver				
	Moisture %	Protein %	Carbohydrate %	Lipid %	Cholesterol %	Moisture %	Protein %	Carbohydrate %	Lipid %	Cholesterol %	Moisture %	Protein %	Carbohydrate %	Lipid %	Cholesterol %
I	76.37 ±1.59	14.79 ±1.22	0.23 ±0.08	1.52 ±0.08	0.33 ±0.08	74.92 ±3.01	17.56 ±1.55	0.44 ±0.10	2.49 ±0.40	0.67 ±0.31	54.18 ±3.57	18.91 ±2.97	2.88 ±0.28	17.71 ±3.98	2.17 ±0.51
II	76.49 ±2.97	16.01 ±1.32	0.51 ±0.12	1.79 ±0.16	0.45 ±0.07	72.50 ±2.55	21.44 ±2.51	0.37 ±0.06	1.84 ±0.46	1.40 ±0.79	53.91 ±1.53	19.06 ±1.35	3.96 ±0.56	20.65 ±1.68	1.34 ±0.51
III	77.33 ±1.85	17.01 ±0.36	0.56 ±0.07	2.11 ±0.16	0.29 ±0.03	78.28 ±5.67	20.25 ±2.77	0.21 ±0.14	1.70 ±0.31	0.54 ±0.05	58.32 ±1.05	18.90 ±3.01	3.05 ±0.15	16.04 ±4.17	1.13 ±0.28
IV	78.03 ±2.10	17.76 ±1.34	0.36 ±0.07	2.84 ±0.46	0.17 ±0.05	78.38 ±1.72	16.31 ±0.62	0.15 ±0.05	1.49 ±0.29	0.31 ±0.08	61.43 ±1.88	18.89 ±1.84	1.72 ±0.46	13.20 ±5.53	1.08 ±0.23
V	81.43 ±1.12	15.33 ±1.29	0.19 ±0.10	1.49 ±0.35	0.10 ±0.01	80.07 ±1.18	14.97 ±0.23	0.11 ±0.04	1.38 ±0.29	0.34 ±0.05	63.67 ±4.11	17.21 ±1.44	1.45 ±0.68	12.85 ±4.73	1.46 ±0.46
VI	78.23 ±1.69	13.61 ±1.26	0.12 ±0.01	1.08 ±0.21	0.10 ±0.00	79.77 ±0.93	15.38 ±1.19	0.11 ±0.01	1.42 ±0.57	0.46 ±0.36	64.45 ±1.03	17.17 ±0.44	1.45 ±0.42	12.66 ±2.25	1.58 ±0.15

PLATE XXVI

Variation in the moisture content of testes, muscle and liver tissues of *Mugil cephalus* during the different stages of maturity.

PLATE XXVI

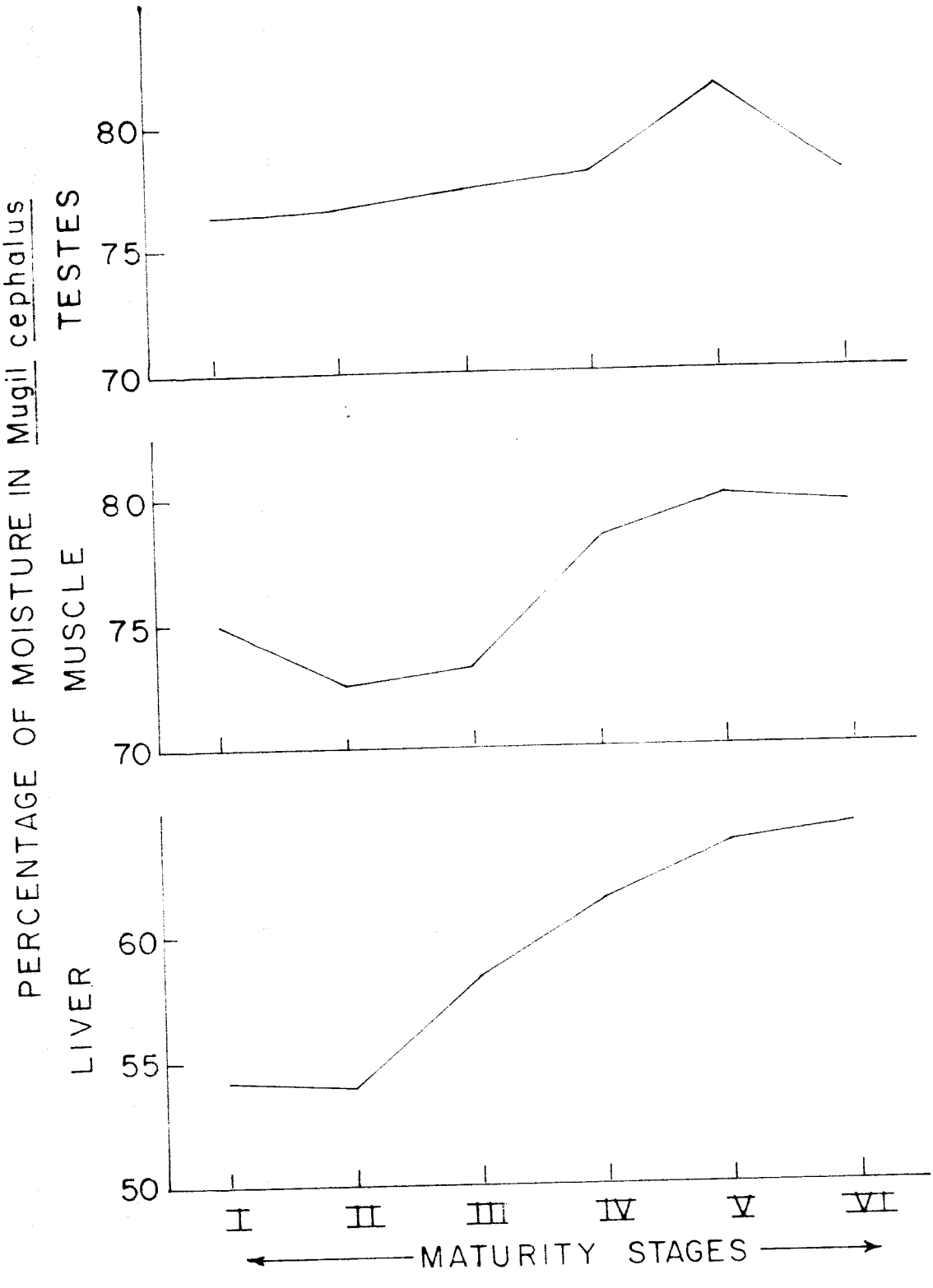

PERCENTAGE OF MOISTURE IN Mugil cephalus
TESTES
80
75
70
MUSCLE
80
75
70
LIVER
60
55
50
I
II
III
IV
V
VI
MATURITY STAGES

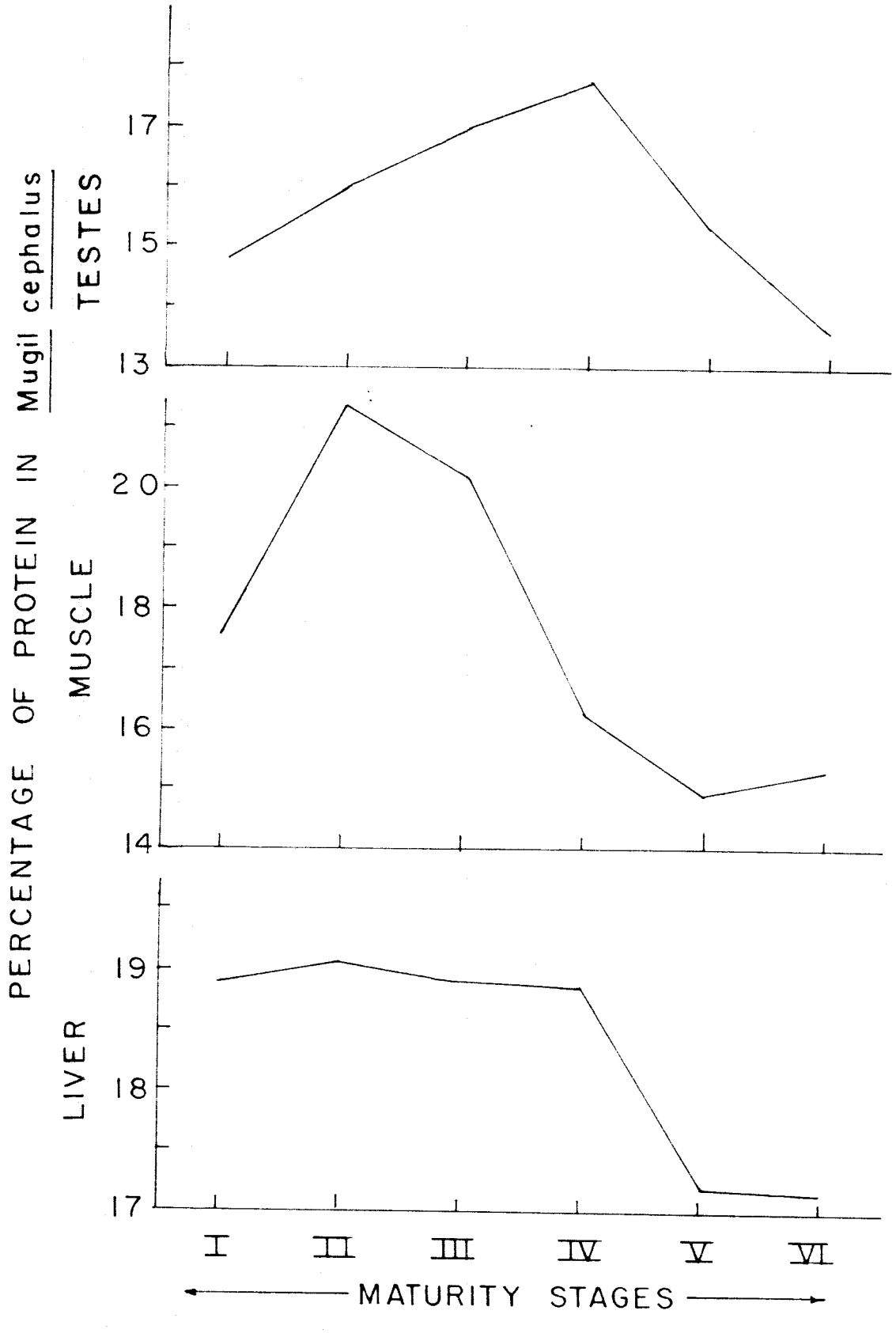
TABLE 9. Biochemical composition of testes, muscle and liver of *M. cephalus* on dry weight basis, during different stages of maturity.

Stages	Testes				Muscle				Liver			
	Protein %	Carbohydrate %	Lipid %	Cholesterol %	Protein %	Carbohydrate %	Lipid %	Cholesterol %	Protein %	Carbohydrate %	Lipid %	Cholesterol %
I	62.59	0.97	6.42	1.41	70.02	1.77	9.92	2.67	41.27	6.29	38.66	4.74
II	68.09	2.16	7.91	1.91	77.97	1.36	6.68	5.08	41.35	8.58	44.80	2.91
III	75.03	2.45	9.30	1.32	75.78	0.82	6.36	2.03	45.35	7.33	38.49	2.71
IV	80.84	1.63	12.92	0.76	75.41	0.71	6.88	1.45	48.97	4.46	34.23	2.79
V	82.55	1.02	8.06	0.52	75.12	0.54	6.90	1.68	47.37	3.98	35.36	4.02
VI	62.52	0.55	4.96	0.45	76.03	0.56	7.04	1.27	48.31	4.08	35.62	4.44

PLATE XXVII

Variation in the protein content of testes, muscle and liver tissues of *Mugil cephalus* during the different stages of maturity.

PLATE XXVII


PERCENTAGE OF PROTEIN IN Mugil cephalus
TESTES
17
15
13
MUSCLE
20
18
16
14
LIVER
19
18
17
I
II
III
IV
V
VI
MATURITY STAGES

Carbohydrate: The carbohydrate content of the testes ranges from 0.12 to 0.56%. It was 0.23% in the immature testes and gradually increased to 0.56% in the third stage of maturity. Thereafter, it decreased gradually to a level of 0.12% in the spent testes (Table 8, Plate XXVIII). On dry weight basis the percentage of the carbohydrate varied from 2.45% in the third stage to 0.55% in the spent stage. The trend of variation of carbohydrates at different maturity stages was almost similar to that of the fresh tissue.

Lipid: As in the case of carbohydrate, the lipid content of the testes showed a gradual increase from 1.52% in the immature stage to 2.64% in the fourth stage (Table 8, Plate XXIX). The minimum value of lipid was observed in the spent stage. Both dry and fresh tissue estimations indicated the same trend, the fifth and sixth stages showing a sharp decrease in lipid content.

Cholesterol: Unlike the above parameters, the cholesterol content of the testes was relatively higher in the first and second stages of maturity. From the highest value observed in the latter stage, it gradually decreased to reach the minimum values in the mature and spent stages (Table 8, Plate XXX).

Liver

Moisture: The moisture content in the liver tissue ranged from 53.91% to 63.67% in different maturity stages.

PLATE XXVIII

Variation in the carbohydrate content of testes, muscle and liver tissue of *Mugil cephalus* during the different stages of maturity.

PLATE XXV

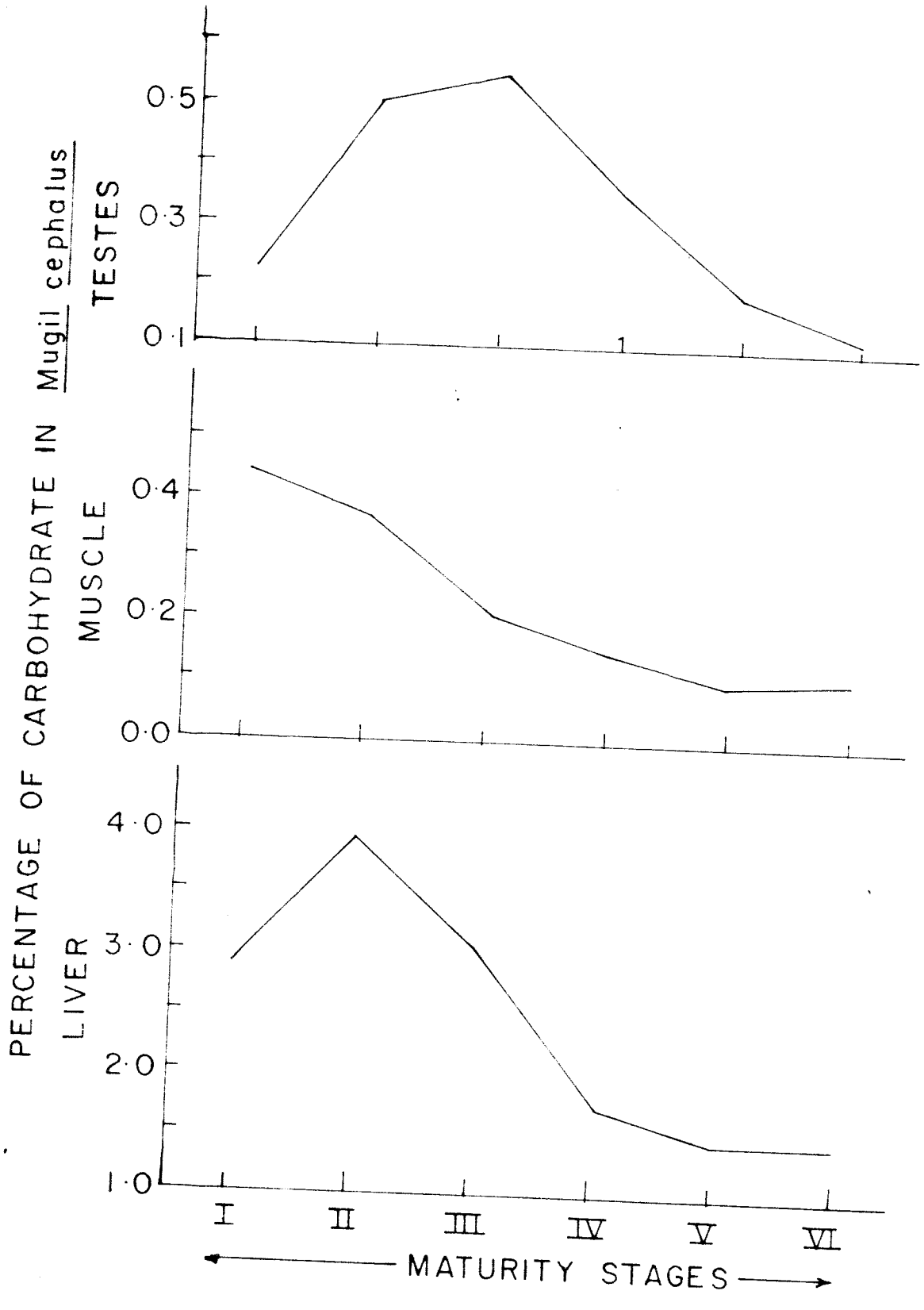

PERCENTAGE OF CARBOHYDRATE IN Mugil cephalus
TESTES
0·5
0·3
0·1
1
MUSCLE
0·4
0·2
0·0
LIVER
4·0
3·0
2·0
1·0
I
II
III
IV
V
VI
MATURITY STAGES

PLATE XXIX

Variation in the lipid content of testes, muscle and liver tissues of Mugil cephalus during the different stages of maturity.

PLATE XXVIII

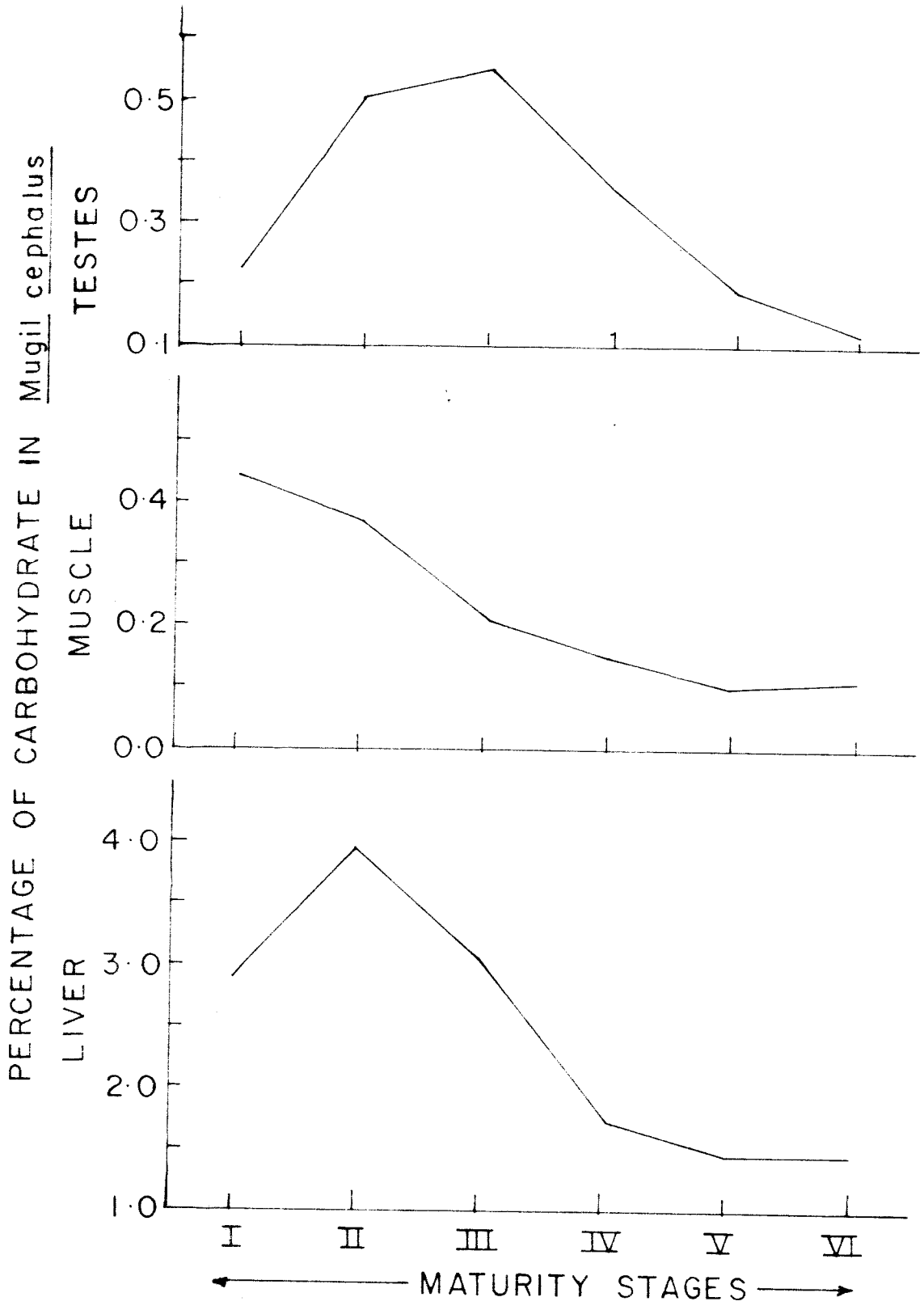


PLATE XXIX

Variation in the lipid content of testes, muscle and liver tissues of Mugil cephalus **during the different stages of maturity.**

PLATE XXIX

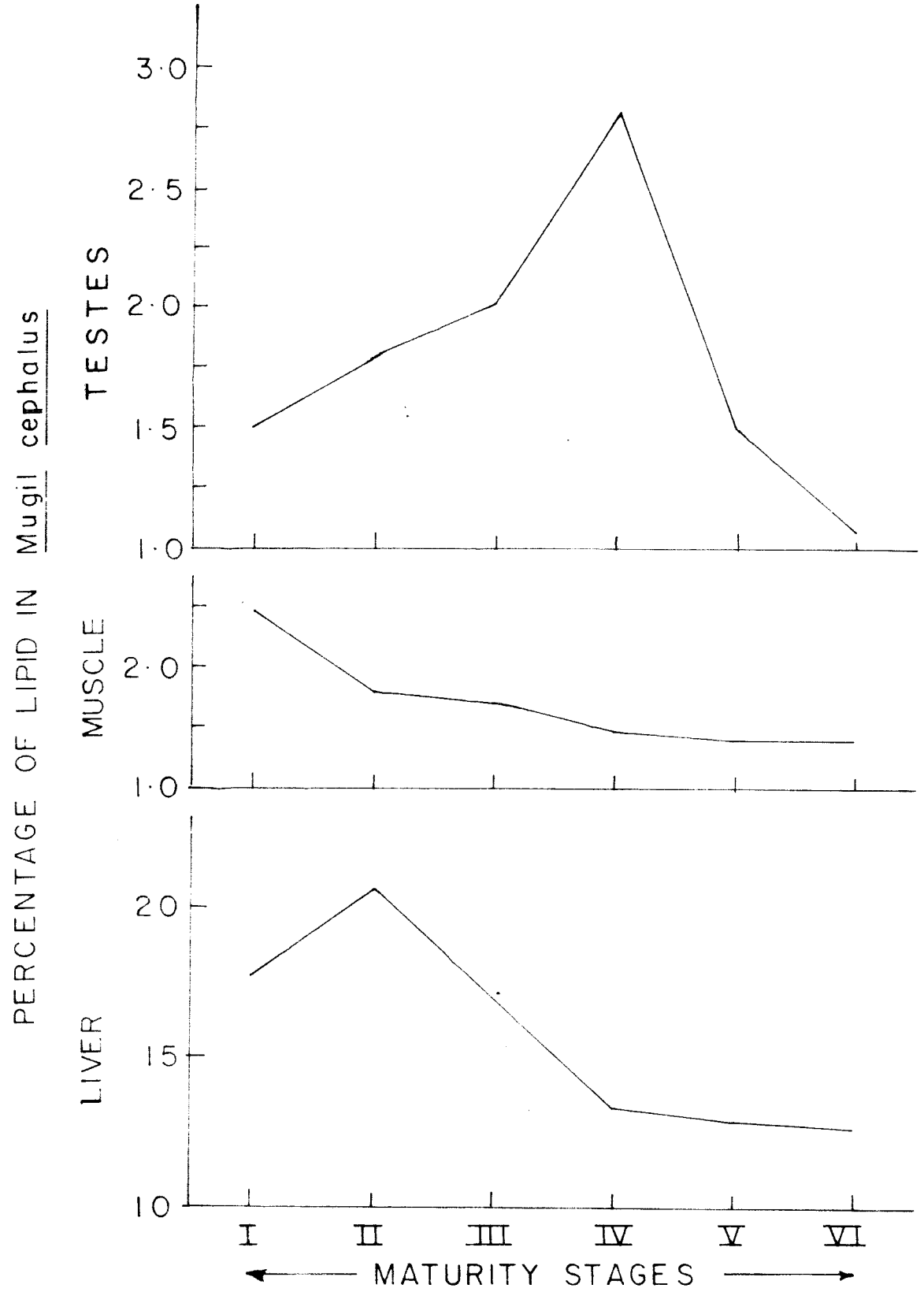
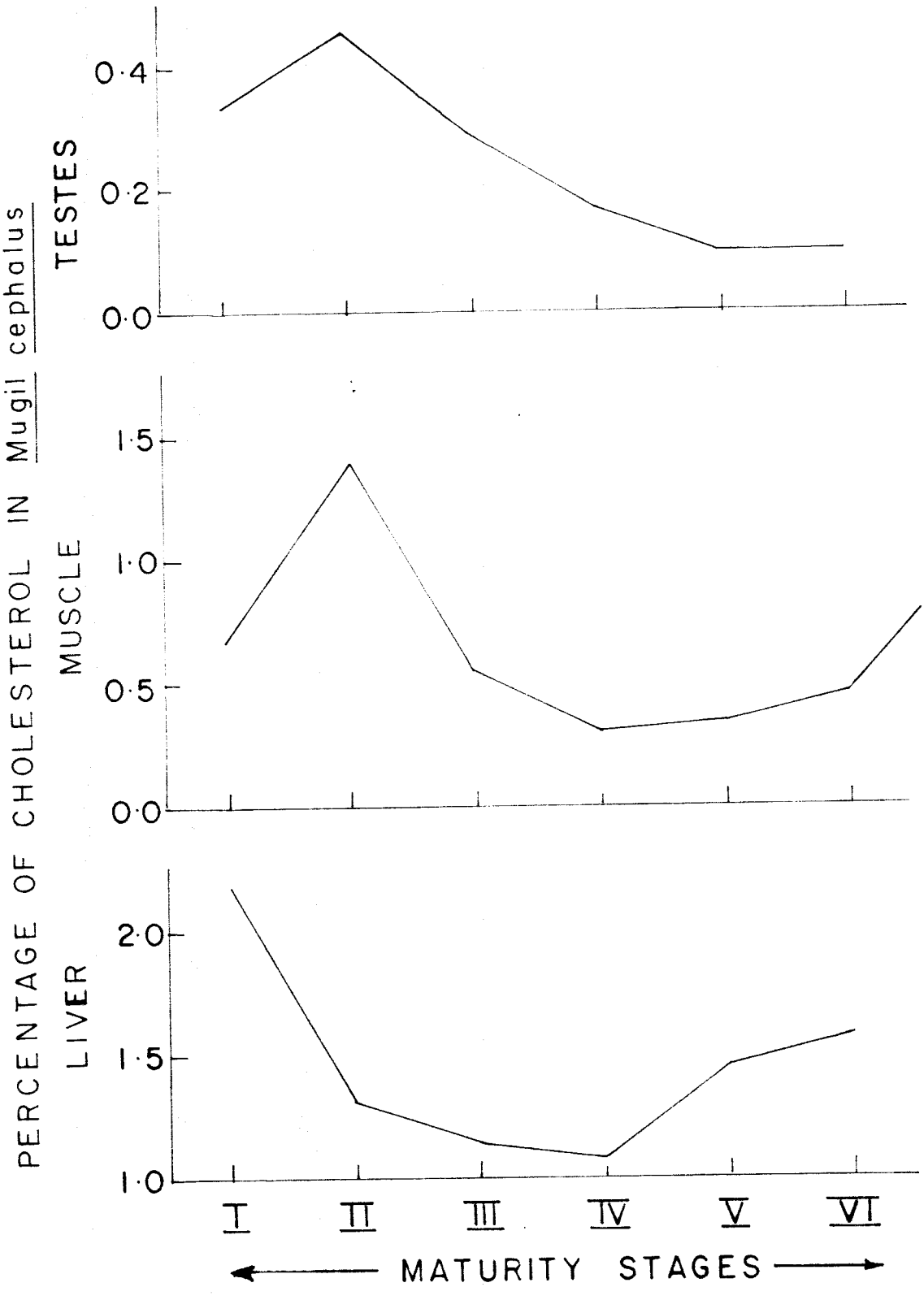

PERCENTAGE OF LIPID IN Mugil cephalus
TESTES
3·0
2·5
2·0
1·5
1·0
MUSCLE
2·0
1·0
LIVER
20
15
10
I
II
III
IV
V
VI
MATURITY STAGES

PLATE XXX

Variations in the cholesterol content of testes, muscle and liver tissues of *Mugil cephalus* during the different stages of maturity.

PLATE XXX


PERCENTAGE OF CHOLESTEROL IN Mugil cephalus
TESTES
0·4
0·2
0·0
MUSCLE
1·5
1·0
0·5
0·0
LIVER
2·0
1·5
1·0
I
II
III
IV
V
VI
MATURITY STAGES

In the immature testes it was 54.18% and this level decreased to 53.91% in the second stage; as to increase again from the next stage (Stage III) to the fourth stage. Although the moisture content in the liver was lower than that in the gonad the distribution pattern in the different stages of maturity was almost similar in these two tissues.

Protein: The percentage variation of the protein content of the liver tissue in the different maturity stages of testes was seen between 17.17 and 19.06%. Protein was found to decrease from the highest value of 19.06% in the second stage of maturity to about 17.17% in the sixth stage (Table 3, Plate XXVII). On dry weight basis, the protein content showed a steady rise from 41.27% in the immature stage to 48.97% in the fourth stage. In the oozing stage the protein content showed a slight decrease. In the spent condition the protein level of the tissue was found to be relatively higher than that in the previous stage.

Carbohydrate: The carbohydrate content in the liver in various stages of testicular maturation was found to range from 1.45 to 3.96%. In the first three stages it was comparatively higher than in the last three stages. From a level of 2.88% in the first stage it increased to 3.96% in the II stage. As the process of maturation advanced from III to IV stage, the carbohydrate

content decreased sharply from 3.05 to 1.72%. Thereafter it remained around 1.45%. Almosta similar pattern of distribution of carbohydrate content was observed on dry weight basis, when it was found to vary from 3.98 to 8.58%. It was also observed that the liver tissue contained relatively higher amount of carbohydrate among the tissues studied at present.

Lipid: The lipid values of the liver tissue in different maturity stages showed a distinct pattern. As in the case of protein, the values were high in the first two maturity stages, while they were considerably lower in the advanced stages. The percentage of lipid content in the liver tissue was highest in the second stage of maturity (20.65%). From this level it decreased by about 4% in the third stage, thereafter showing a gradual decrease upto the sixth stage of maturity (12.66%). The lipid content showed an initial rise from the first stage (17.71%) to the second stage (20.65%). On the dry weight basis, the minimum percentage of lipid in the liver tissue was recorded in the fourth stage of maturity.

Cholesterol: The cholesterol level of the liver showed a decline from the first stage to the fourth stage of maturity. The highest value of 2.17% was recorded in the first stage and the lowest 1.08%, in the fourth stage. From this low level, it was found to increase slightly in the fifth and sixth stages of maturity (Table 8, Plate XXX).

On the dry weight basis although the cholesterol content in the different stages showed a similar pattern of change, the lowest value was observed in the third stage.

Muscle

Moisture: The moisture level in the muscle tissue of M. cephalus at different maturity stages was comparable to that in the testes. It showed wide fluctuation in the immature and early maturing stages (Table 8, Plate XXVI), although the mean values were ranging between 72.50 and 74.92%. The lowest value (72.50%) was recorded in the second stage. In the subsequent maturity stages the moisture level was found to increase, attaining the highest level of 80.07% in the fifth stage. In the spent condition, however, the muscle tissue contained slightly lower level of moisture.

Protein: Muscle protein was found to build up from 17.56% in the first stage to 21.44% in the second stage. A gradual decline was then noticed up to the fifth stage (14.97%) followed by a slight rise in the sixth stage (Table 8, Plate XXVII). On dry weight basis, the minimum value or muscle protein was observed in the first stage (Table 9).

Carbohydrate: During maturation of the testes, the carbohydrates in the muscle showed a steady decline from

the highest value of 0.44% in the first stage upto the lowest (0.11%) in the fifth and sixth stages, (Table 8, Plate XXVIII). The variation of carbohydrates in the muscle during different maturity stages obtained from the data calculated on dry weight basis showed a more or less similar pattern of distribution.

Lipid: The lipid content of the muscle tissue of M. cephalus varied between 1.38 and 2.49% in different maturity stages and the values were found to be comparable to those of the testes. The highest value of 2.49% was observed in the first stage. From this level, it continued to decrease upto the fifth stage. As in the case of the testes the lipid content of the muscle was found to increase in the spent fishes. On the dry weight basis, the lipid level of the muscle fluctuated between 6.36 and 9.92%. In the maturing stages the lipid content was found to be lower than in the immature and spent stages.

Cholesterol: During maturation of the testes the cholesterol level of the muscle tissue showed an initial increase from the immature to second stage and then a decrease in the third and fourth stages (Table 8, Plate XXX). Between the last two stages a slight increase was observed. The maximum value (1.40%) was recorded in stage II and the minimum (0.31%) in the stage IV. The data of cholesterol content in the dry tissue showed a

similar pattern of fluctuation in different stages of maturity.

L. parsia

Testes

Moisture: In the immature testes the moisture content of the tissue was 77.50%. As the maturation of the testes advanced through different stages, the moisture content also increased and attained the maximum value of 81.32% in the fifth stage. In the spent testes, the moisture level was of the value of 78.19% .

Protein: In the fresh tissue analysis, the gonadal protein was found to range from 15.14% to 18.79% during the different maturity stages. While on a dry weight basis it was found to vary between 67.29% and 83.99%. As in the case of M. cephalus, the protein content in the testes of L. parsia was found to increase with the progress of maturation (Table 10, Plate XXXII). In the mature condition, the testes had about 18.53% protein. In the spent stages, unlike in M. cephalus, the protein level showed a slight increase. In dry tissue, the protein range was between 83 and 84% in the third, fourth and fifth stages. In the spent condition it showed a reduction by about 5%.

Carbohydrate: The carbohydrate content of the testes showed a slight increase from 0.24% in the first

TABLE 10. Biochemical composition of fresh tissues of testes, muscle and liver of *L. parsia* male during the different stages of maturity.

Stages	Testes					Muscle					Liver				
	Moisture %	Protein %	Carbohydrate %	Lipid %	Cholesterol %	Moisture %	Protein %	Carbohydrate %	Lipid %	Cholesterol %	Moisture %	Protein %	Carbohydrate %	Lipid %	Cholesterol %
I	77.50 ±0.95	15.14 ±0.64	0.24 ±0.04	1.18 ±0.10	0.30 ±0.05	74.72 ±1.43	20.79 ±1.11	0.47 ±0.06	1.73 ±0.07	0.77 ±0.08	66.39 ±0.62	18.91 ±0.73	5.45 ±0.56	5.84 ±0.39	1.68 ±0.11
II	76.92 ±0.84	16.82 ±0.66	0.46 ±0.32	1.29 ±0.11	0.28 ±0.05	75.35 ±0.75	21.01 ±4.79	0.23 ±0.04	1.13 ±0.09	0.55 ±0.04	65.23 ±0.62	20.87 ±0.88	5.89 ±0.32	6.22 ±0.44	1.85 ±0.06
III	77.42 ±1.41	18.79 ±0.93	0.45 ±0.05	1.35 ±0.11	0.22 ±0.01	75.64 ±0.45	20.17 ±0.56	0.15 ±0.01	0.91 ±0.06	0.49 ±0.04	68.24 ±0.75	19.42 ±0.41	4.74 ±0.28	5.33 ±0.28	1.05 ±0.19
IV	77.94 ±1.45	18.53 ±1.62	0.23 ±0.05	1.56 ±0.08	0.16 ±0.02	75.83 ±0.41	19.05 ±0.84	0.13 ±0.01	0.65 ±0.04	0.26 ±0.04	70.08 ±0.42	18.77 ±0.42	3.57 ±0.44	3.91 ±0.42	0.79 ±0.04
V	81.32 ±0.93	15.68 ±1.05	0.18 ±0.02	1.29 ±0.09	0.11 ±0.01	76.18 ±0.47	18.43 ±0.41	0.09 ±0.01	0.45 ±0.03	0.18 ±0.02	72.11 ±0.52	17.61 ±0.44	2.38 ±0.61	2.99 ±0.32	0.69 ±0.04
VI	78.19 ±1.29	16.66 ±0.83	0.14 ±0.01	1.12 ±0.08	0.09 ±0.01	75.01 ±0.63	18.76 ±0.34	0.11 ±0.01	0.47 ±0.02	0.19 ±0.01	70.89 ±0.72	18.37 ±0.32	1.85 ±0.39	2.93 ±0.38	0.65 ±0.03

PLATE XXXI

Variation in the moisture content of testes, muscle and liver tissues of *Liza parsia* during the different stages of maturity.

PLATE XXXI

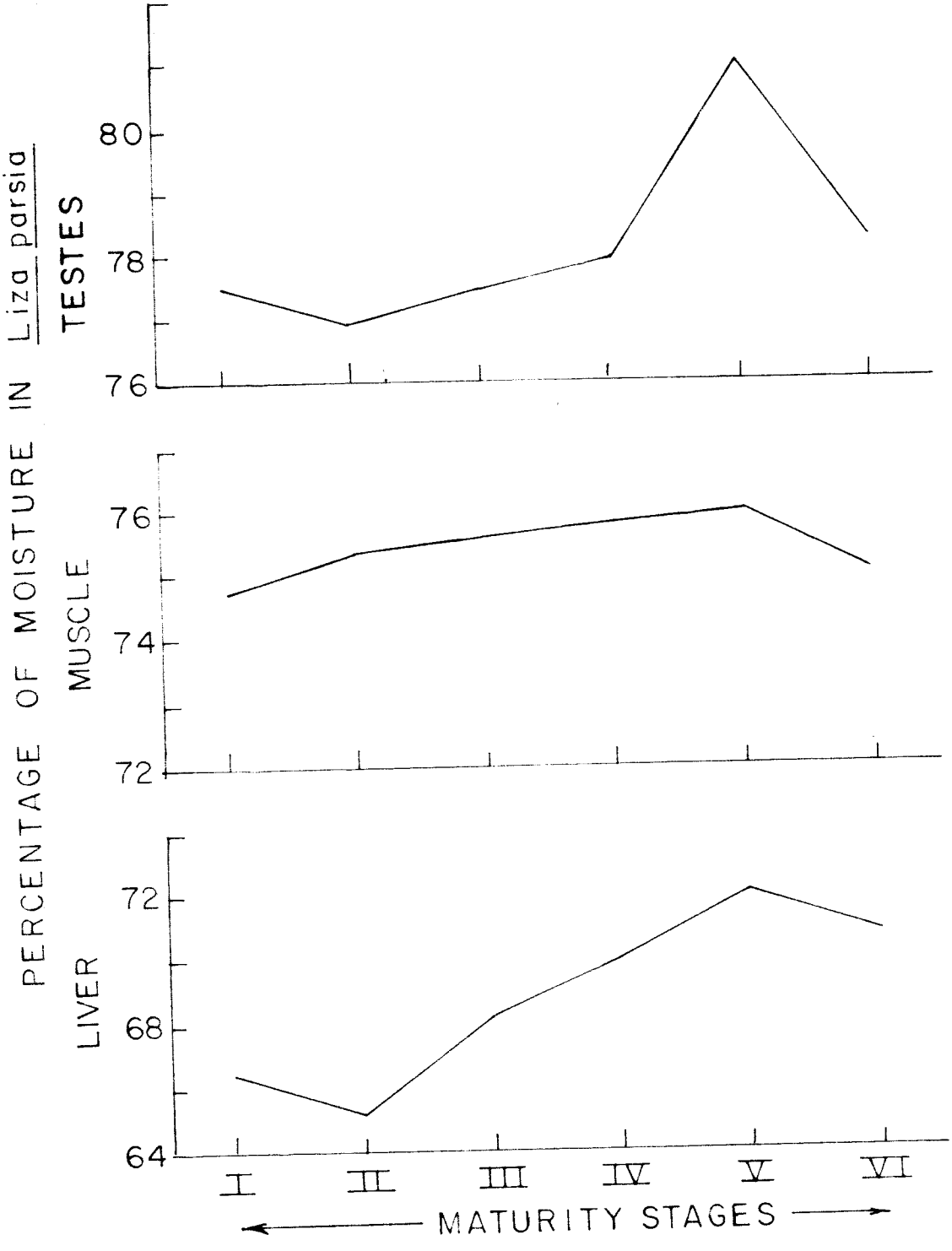

PERCENTAGE OF MOISTURE IN Liza parsia
TESTES
80
78
76
MUSCLE
76
74
72
LIVER
72
68
64
I
II
III
IV
V
VI
MATURITY STAGES

PLATE XXXII

Variation in the protein content of testes, muscle and liver tissues of Liza parsia during the different stages of maturity.

PLATE XXXII

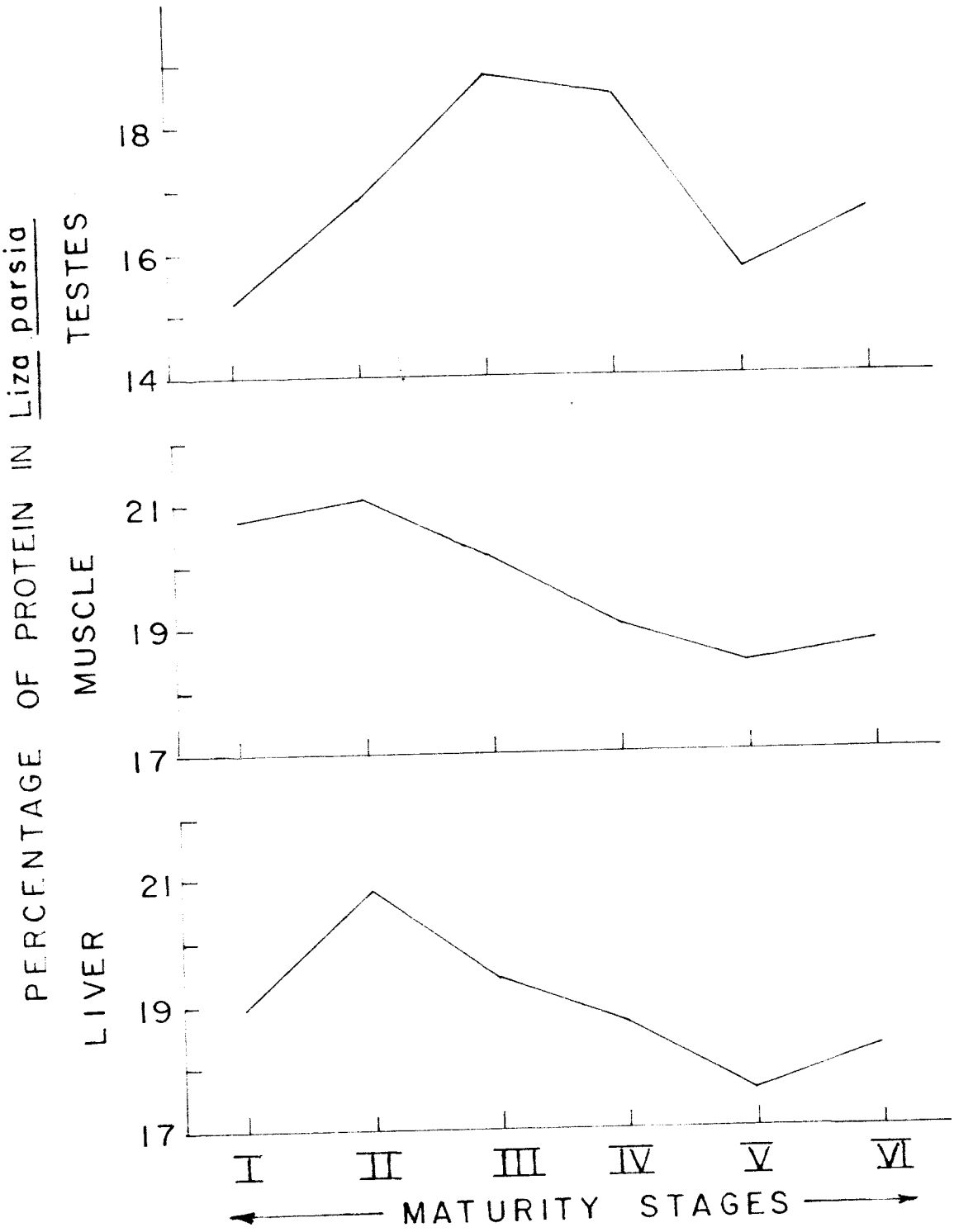
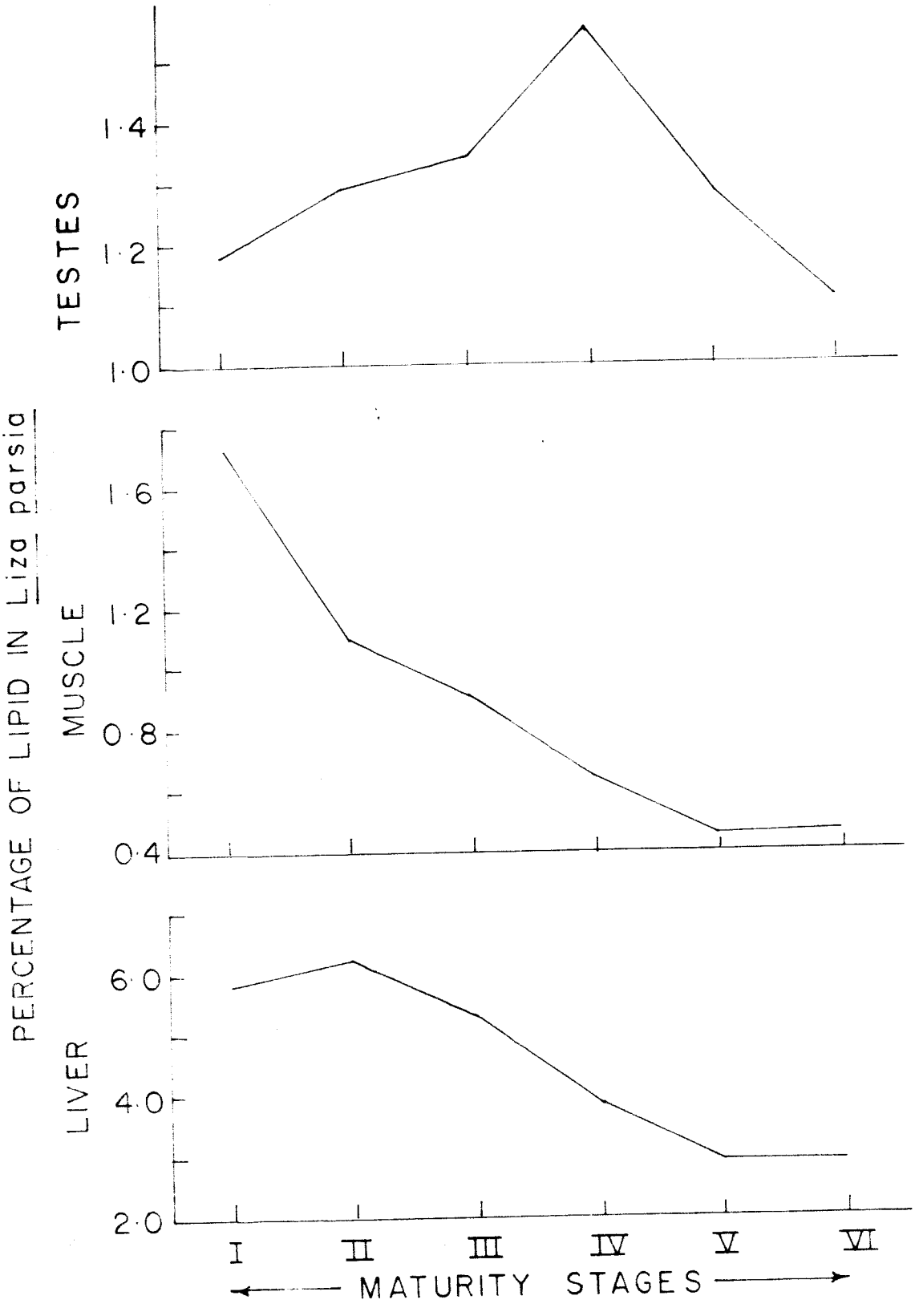

PERCENTAGE OF PROTEIN IN Liza parsia
TESTES
18
16
14
MUSCLE
21
19
17
LIVER
21
19
17
I
II
III
IV
V
VI
MATURITY STAGES

PLATE XXXIV

variation in the lipid content of testes, muscle and liver tissues of *Liza parsia* during the different stages of maturity.

PLATE XXXIV


PERCENTAGE OF LIPID IN Liza parsia
TESTES
1·4
1·2
1·0
MUSCLE
1·6
1·2
0·8
0·4
LIVER
6·0
4·0
2·0
I
II
III
IV
V
VI
MATURITY STAGES

stage to the maximum value of 0.46% in the second stage. This was followed by a steady decline in the subsequent maturity stages, reaching the minimum value of 0.14% in the spent stage (Table 10, Plate XXXIII). A similar pattern of distribution of carbohydrates was observed on the dry weight basis also, with the values varying between 0.65 and 1.99%.

Lipid: The lipid level in the testes of L. parsia was less than 2% unlike in M. cephalus where it was found above 2% in the late maturing stages. However, the general trend of lipid level in different maturity stages was more or less similar in these two fishes. As in M. cephalus a gradual rise in the percentage of gonadal lipids was observed from the first stage (1.18%) to the fourth stage (1.56%) (Table 10, Plate XXXIV).

This was followed by a decline in the fifth and sixth stages. The minimum value was recorded in the spent stage (1.12%). A similar trend was observed on the dry weight basis (Table 11) where it was found varying between 5.13 and 7.06%.

Cholesterol: The cholesterol content of the testes tissue showed a steady rate of depletion from the first stage (0.30%) to the sixth stage (0.09%) (Table 10, Plate XXXV). The initial rise in this component observed in M. cephalus was not seen in L. parsia. However, the

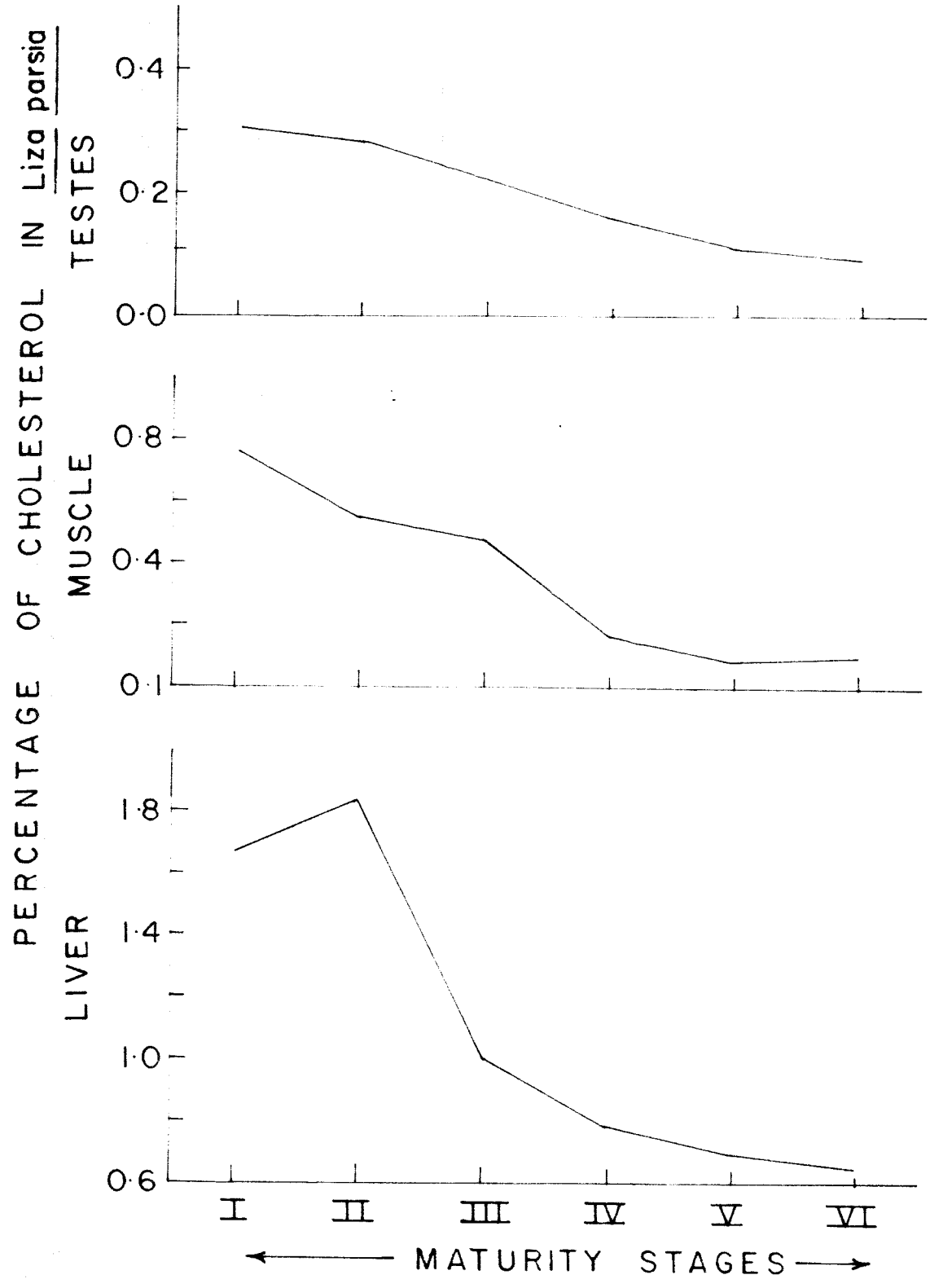
TABLE 11. Biochemical composition of testes, muscle and liver tissues of *L. parsia* on dry weight basis

Stages	Testes				Muscle				Liver			
	Protein	Carbohydrate	Lipid	Cholesterol	Protein	Carbohydrate	Lipid	Cholesterol	Protein	Carbohydrate	Lipid	Cholesterol
	%	%	%	%	%	%	%	%	%	%	%	%
I	67.29	1.07	5.26	1.34	82.23	1.86	6.85	3.04	56.26	16.21	17.39	4.99
II	72.86	1.99	5.63	1.22	85.24	0.91	4.56	2.24	60.02	16.93	17.89	5.31
III	83.21	1.98	5.96	0.98	82.82	0.63	3.75	1.99	61.15	14.93	16.77	3.29
IV	83.99	1.06	7.06	0.74	78.80	0.53	2.67	1.08	62.73	11.93	13.07	2.64
V	83.92	0.94	6.95	0.59	77.36	0.39	1.89	0.77	63.12	8.54	10.71	2.49
VI	76.40	0.65	9.13	0.43	75.09	0.42	1.89	0.75	61.47	6.19	9.81	2.18

PLATE XXXV

Variation in the cholesterol content of the testes, muscle and liver tissues of *Liza parsia* during the different stages of maturity.

PLATE XXXV


PERCENTAGE OF CHOLESTEROL IN Liza parsia
TESTES
0·4
0·2
0·0
MUSCLE
0·8
0·4
0·1
LIVER
1·8
1·4
1·0
0·6
I
II
III
IV
V
VI
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percentage distribution was comparable in the two species.

Liver

Moisture: After an initial decline from first stage (66.39%) to the second stage (65.23%) the percentage of moisture in the liver tissue showed a steady rise upto the fifth stage (72.11%). In the spent stage, the moisture content of the liver tissue slightly decreased (Table 10, Plate XXXI).

Protein: The protein level in the liver of L. parsia and M. cephalus was more or less similar. The percentage of protein increased from 18.91% in the first stage to 20.87% in the second stage and thereafter decreased to 17.61% in the fifth stage (Table 10, Plate XXXII). However on dry weight basis, a steady rise in protein content was observed upto the fifth stage, and then a decline in the spent condition (Table 11).

Carbohydrate: From 5.45% in the first stage, the carbohydrate content of liver tissue in L. parsia increased to 5.89% in the second stage. In the subsequent stages it showed a gradual decline to reach 1.85% in the sixth stage (Table 10, Plate XXXIII). On dry weight basis too a similar trend was observed. A comparison of the carbohydrate level in the liver of M. cephalus and L. parsia showed that it was relatively higher in the latter species.

Lipid: As in the case of M. cephalus the highest value of lipid content (6.22%) was observed in the second stage. With the progress of maturation of testes, it decreased gradually to reach the minimum value of 2.93% in the sixth stage. On dry weight basis, the lipid values of the liver varied from 9.81% in the sixth stage to 17.89% in the second stage. As compared with the lipid values in the liver of M. cephalus it was observed that in L. parsia the lipid level was considerably less both on the fresh weight and dry weight basis.

Cholesterol: The cholesterol level in the liver of L. parsia showed a similar pattern of fluctuation as that of lipid, with a slight increase from the first (1.67%) to the second stage (1.85%) followed by a gradual decline upto the sixth stage (0.65%), (Table 10, Plate XXXV). A similar distribution trend was reported in the cholesterol content in the dry tissue although values were higher and ranged from 2.18% to 5.31%.

Muscle

Moisture: The percentage of moisture in the muscle tissue as observed in the testes and liver followed the same general trend. It increased steadily from stage I (74.72%) to Stage V (76.18%) and in Stage VI it showed a slight decline (Table 10, Plate XXXI).

Protein: The protein content of the muscle tissue during spermatogenesis showed an initial increase from 20.79% in the first stage to 21.01% in the second stage. Thereafter a depletion was observed through the different maturity stages, reaching a minimum value of 18.43% in the fifth stage. A slight increase in protein content was recorded in the sixth stage. On dry weight basis this apparent increase of protein content in the sixth stage was not observed. On the contrary, protein level was found to be the lowest in this stage.

Carbohydrate: The maximum value of carbohydrate in muscle was recorded in Stage I (0.47%). With the advancement of maturity, the value declined and reached the minimum in the Stage V (0.09%). However, a slight increase was observed in the VI stage (0.11%),(Table 10, Plate XXXIII). The pattern of carbohydrate distribution in the wet and dry samples during different stages of maturity was almost similar.

Lipid: From the highest value of 1.73% in the first stage, the lipid content showed a decline at each maturity stage and the minimum level of 0.45% was observed in the fifth stage. On dry weight basis the least amount was recorded in the sixth stage.

Cholesterol: The muscle cholesterol content was found to be higher than that in the testes but lower than

that in the liver. In L. parsia it was highest in Stage I. As in the case of lipid, cholesterol also showed a gradual decrease with the maturation of the testes. The lowest amount was recorded in the fifth stage in fresh tissue and in the sixth stage in the dry tissue (Tables 10 and 11, Plate XXXV).

The results of the biochemical analysis of the testes, liver and muscle of M. cephalus and L. parsia during the maturation process of testes, indicated that the basic pattern of change in the biochemical composition was more or less similar in both the species. Among the tissues, the testes indicated an increase of all the constituents with the progress of maturation from the immature testes to the mature condition, while the liver and the muscle showed a partial depletion of all the components except water. An increase in water content was noticed in all the tissues with the progress of maturation.

The data obtained was subjected to statistical analysis. The analysis of variance was performed for each biochemical parameter in each species to test if there was any significant variation in the biochemical parameters (i) between the stages and (ii) between the tissues in each stage. Since the interaction between the two sources of variation (stage and tissue) was significant, the main effects were tested against the interaction. The analysis of variance of all the parameters except moisture indicated that there was

no significant variation between the stages while between the tissues the variation was significant at both 5% and 1% levels. Variations in moisture were significant between the stages and between the tissues at both 5% and 1% levels (Tables13 and 14).

Serum analysis in M. Cephalus

Estimation of biochemical parameters such as protein, carbohydrate, lipid and cholesterol were also made in the blood serum of M. Cephalus in the different stages of maturity (Table 12,Plate XXXVI). Protein content was found to vary between 7.35 and 9.07g/100 ml the maximum values being observed in the first two stages and the minimum in the fourth stage. The carbohydrate content increased from 82.37 mg/100 ml in the first stage to 108.63 mg/100 ml in the third stage. Thereafter it decreased gradually to 80.85 mg/100 ml in the sixth stage. The lipid content indicated an alternating pattern of increase and decrease upto the fourth stage. One of the peaks of increase was observed in the second stage (1.18 g/100 ml) and the second one in the fourth stage (0.89 g/100 ml). The lowest value was recorded in the sixth stage (0.52 g/ 100 ml). The cholesterol content of the blood serum indicated a sharp decline from the first stage (294.54 mg/ 100 ml) to fifth stage (155.72 mg/100 ml) but increased to a level of 246.81 mg/100 ml in the sixth stage.

TABLE 12. Biochemical analysis of the serum of *M. cephalus* during different stages of maturity.

Stage	Protein(g/100 ml)	Carbohydrate (mg/100 ml)	Lipid(g/100 ml)	Cholesterol(mg/100 ml)
I	9.01 ± 1.48	82.37 ± 19.42	0.80 ± 0.66	294.54 ± 81.31
II	9.07 ± 0.18	87.60 ± 15.80	1.18 ± 0.23	239.17 ± 48.21
III	8.64 ± 0.86	108.63 ± 15.80	0.65 ± 0.17	220.24 ± 17.90
IV	7.35 ± 0.35	97.27 ± 6.74	0.89 ± 0.15	170.94 ± 19.79
V	7.34 ± 0.61	90.06 ± 4.29	0.69 ± 0.41	155.72 ± 13.88
VI	8.04 ± 0.33	80.85 ± 3.96	0.52 ± 0.11	246.81 ± 37.09

PLATE XXXVI

Biochemical composition of the serum of male *Mugil cephalus* during different maturity stages.

PLATE XXXVI

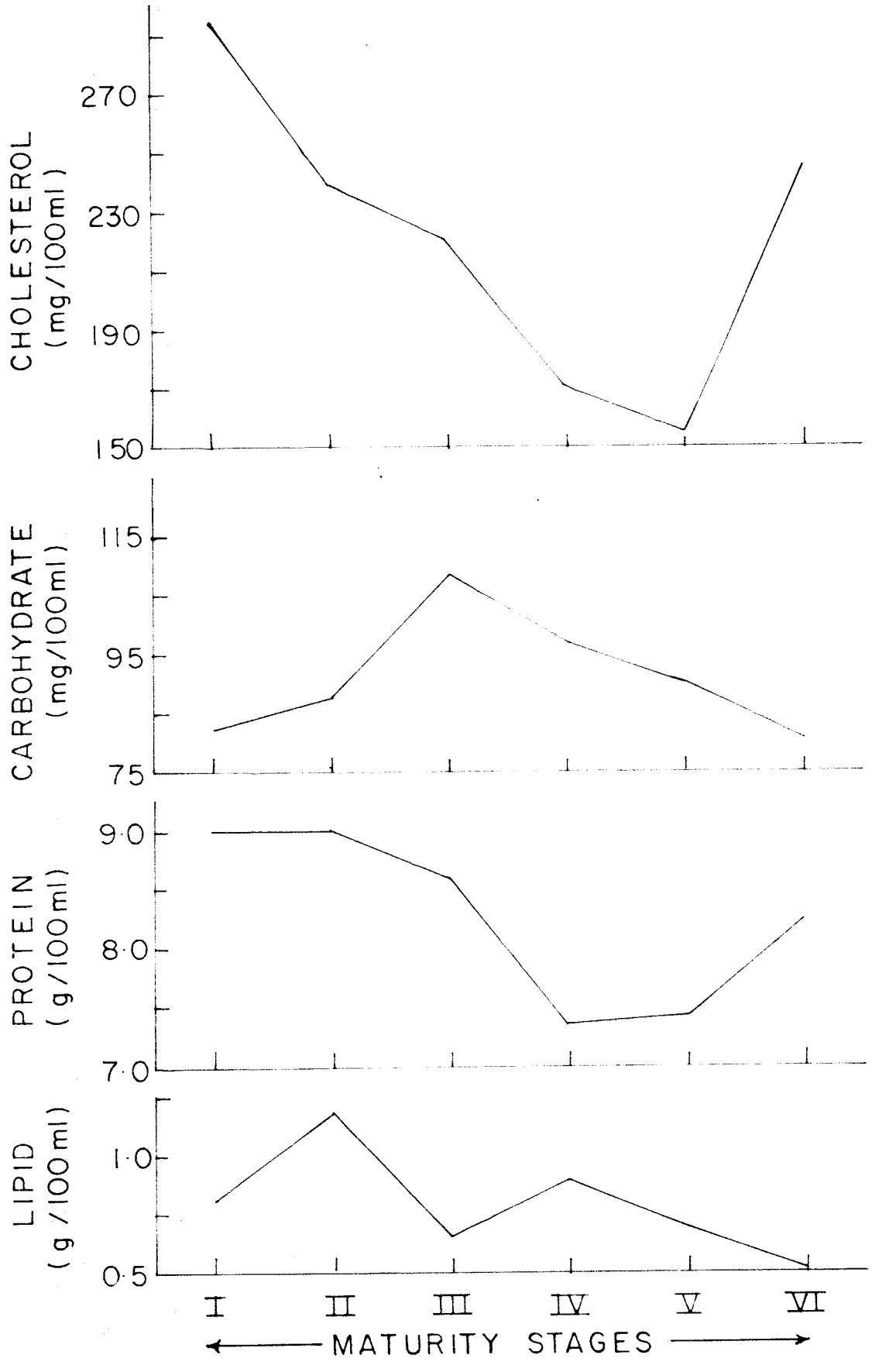
CHOLESTEROL (mg/100ml)
270
230
190
150
CARBOHYDRATE (mg/100ml)
115
95
75
PROTEIN (g/100ml)
9·0
8·0
7·0
LIPID (g/100ml)
1·0
0·5
I
II
III
IV
V
VI
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TABLE 13. Analysis of variance of the Biochemical parameters in Mugil cephalus

	Source of variation	df	S.S.	M.S	F
Moisture	Stage	5	2732.651	546.530	11.561*
	Tissue	2	25812.300	12906.150	273.014*
	Interaction	10	472.728	47.272	7.316
	Errors	297	1919.047	6.461	-
Protein	Stage	5	382.570	76.514	1.760
	Tissue	2	400.540	200.270	4.610
	Interaction	10	434.290	43.429	13.500
	Errors	297	954.950	3.210	-
Carbo-hydrate	Stage	5	49.210	9.840	1.840
	Tissue	2	382.910	191.450	35.960
	Interaction	10	53.230	5.323	57.236
	Errors	297	27.760	0.093	-
Lipid	Stage	5	386.710	77.340	1.090
	Tissue	2	15080.670	7540.340	106.940
	Interaction	10	705.150	70.515	13.025
	Errors	297	1607.880	5.413	-
Cholesterol	Stage	5	14.260	2.850	2.330
	Tissue	2	92.740	46.370	38.000
	Interaction	10	12.240	1.224	13.191
	Errors	297	27.558	0.092	-

* Significant at 5% and 1% levels

TABLE 14. Analysis of variance of the Biochemical parameters in *Liza parsia*.

	Source of variation	df	S.S	M.S	F
Moisture	Stage	5	4042.279	808.455	72.038*
	Tissue	2	6134.739	3067.369	273.323*
	Interaction	10	112.225	11.222	4.117
	Errors	357	973.021	2.725	-
Protein	Stage	5	284.634	56.926	2.099
	Tissue	2	575.695	287.847	10.618
	Interaction	10	271.086	27.108	40.334
	Errors	357	289.927	0.672	-
Carbo-hydrate	Stage	5	119.860	23.972	1.422
	Tissue	2	1182.354	591.177	35.089
	Interaction	10	168.475	16.847	228.595
	Errors	357	26.304	0.073	-
Lipid	Stage	5	123.549	24.709	2.074
	Tissue	2	989.227	494.613	41.531
	Interaction	10	119.093	11.909	217.323
	Errors	357	19.571	0.054	-
Cholesterol	Stage	5	23.502	4.700	4.298
	Tissue	2	58.672	29.336	26.830
	Interaction	10	10.934	1.093	223.142
	Errors	357	1.746	0.004	-

* Significant at 5% and 1% levels.

DISCUSSION

Reproductive effort in fishes involves three important aspects, namely, pre-zygotic maturation bio-synthesis and storage within the gametes, anabolism of secondary sexual characters and catabolism of reproductive behaviour (Miller, 1984). Several studies are now available on the control mechanisms, underlying the activation of gametogenesis by endogenous factors which initiate the changes and exogenous factors which determine when the endogenous factors become functional (Stacey, 1984). It is also known that the gonad maturation in fishes involves a cyclical demand for material and energy translocated mainly from the somatic source. The changes presented in the major biochemical components in the testis, liver, body muscle and blood serum in the foregoing account show the range, interaction, allocation and utilization of water, protein, lipid and carbohydrate during the different stages of spermatogenesis in M. cephalus and L. parsia.

Moisture forms the major constituent of the soft parts of the body of the fish. Its fluctuation in the body tissues, besides being influenced by the environmental factors, osmotic properties of the cells and the age and growth of the fish(Parker and Vanstone, 1966; Marias and Erasmus, 1977) serves as an indicator of accumulation or decline of metabolic components in the tissues or cells.

The proximate composition of mullets has indicated that about 75.77% of their body weight is formed of water (Mukundan et al., 1981). The moisture content in the body muscles of M. cephalus and L. parsia in the present study also shows it to be between 75 and 80%. Of the three tissues analysed in these species, liver has relatively lower amount of moisture probably because it forms the storage organ for lipid, protein and carbohydrate. With the advancement of maturation, however, an increase in the moisture content of the liver is observed in both the species. This increase as well as the simultaneous decrease of protein, carbohydrate and lipid components in the liver and the rise of these in the testes indicate the depletion of these components from the liver for gonadal maturation and their replacement by the water. The moisture content in the liver tissue of M. cephalus is more or less steady upto the second stage of maturity and thereafter it increases appreciably pointing out that the active depletion of the tissue components in the species starts after the second stage and becomes pronounced in the subsequent stages. In the case of L. parsia, a a decrease in the moisture content is recorded from the first to the second stage, suggesting building up of resources in the liver tissue. Increase in moisture content is noticed only after this stage coinciding with the depletion of other biochemical components from the liver and building up of the generative tissues in the

gonad. The spent stage records a slight decrease in the water content of the liver tissue indicating the commencement of rebuilding of the resources after the release of the reproductive material.

The pattern of change in the moisture content of the muscle tissue in M. cephalus and L. parsia is found to be different. In the former species, there is an initial decrease in the moisture content from the first to the second stage followed by a slight rise in the third stage. An appreciable increase in the moisture level is seen in the subsequent two stages and then it declines in the last stage. This pattern clearly shows that in the muscle tissue of this species, translocation of components starts only after the second stage and that until then, the body resources are actually being built up. Estimation of the percentage composition of the different components of the tissue on a dry weight basis has indicated that in the maturity stages IV and V there is very little fluctuation in the biochemical components, hence it may be concluded that the sudden rise in the moisture content observed in the stages IV and V is not only due to depletion, but also due to positive hydration of the tissue. In L. parsia on the other hand, there is only a slight but steady rise in the water content from the first to the fifth stage, suggesting a gradual depletion of the tissue components throughout the maturity stages. Further, no significant hydration of muscle tissue is seen in the

fourth and fifth stages in this species as observed in M. cephalus.

As compared to the moisture level in the liver and muscle tissue, relatively higher level is seen in the testes of both the species. While in M. cephalus, a gradual increase in the moisture content is observed from the first to the fourth stage, a reverse condition it seen in L. parsia in the first two stages and thereafter an increase in the third and fourth stages.

In both the species an appreciable increase in water content is observed after the fourth stage. This rise in the water content in these stages, however is not accompanied by any significant depletion in the biochemical composition of the tissues indicating that hydration is a characteristic feature of the last stage of maturation. Unlike in the muscle and the liver, where a depletion of the biochemical components is observed with the advancement of maturity stages, in the testes , protein, carbohydrate and lipid resources are observed to build up as the maturation process advances. This suggests that hydration and accumulation of resources could take place simultaneously as two independent events. The increase of water in the final stage of maturation might be for facilitating the formation of free running milt during spawning.

Next to water, protein forms the major component in the biochemical composition of the tissues. Its complex role during the different phases of growth and reproduction in fishes has been discussed by several workers. Hickling (1930), Love and Robertson (1967) and Iles (1974) have shown that the protein synthesised and accumulated in the somatic tissues during the prematuration period is utilised for gamete formation in addition to the growth of the fish. Further, Korzenko (1966) and Love (1970) suggest that certain aminoacids from the muscle may be mobilised for the production of sex products.

Commenting on the increase of collagen content during spawning in fishes such as Clupea pallasii, Mc Bride et al. (1960), have opined that much of the gonad tissue is built from protein from the muscles, and the aminoacids which are not needed by the gonad are converted into collagen and deposited in the skin, jaw extension and in the humped back of the salmon species. In the present study, the protein in the muscle and the liver is found at relatively higher levels in the immature and the early maturing stages. The muscle tissue of both M. cephalus and L. parsia and the liver tissue of L. parsia indicate a sharp rise in the protein level from the first stage to the second stage, but in the case of the liver tissue of M. cephalus, only a slight rise in the protein level is observed. As the maturation of testes advances, the

protein level decreases in both these tissues while it shows a steady increase in the testes. This pattern of distribution of protein level suggests that the protein gets accumulated in the somatic tissues (muscle and liver) in the immature and in the very early stages of maturation and subsequently it gets translocated to the testes, as spermatogenesis progresses. The maximum level of muscle protein in M. cephalus has been 21.44% and that in L. parsia 21.01%, while the minimum levels in these species are seen at 14.97% and 18.42% respectively. This indicates that the depletion from muscle protein is more pronounced in M. cephalus than in L. parsia. However, such a difference in the liver tissues of the two species is not observed, as the maximum and minimum levels of protein are more or less in the same range in the two species indicating a comparable degree of depletion.

The carbohydrate stored in the form of glycogen contributes to about 0.5–1.5% of the body composition of the fish. But as it is readily mobilized, highly reducible and liberates energy under anaerobic conditions, it forms an important source of energy (Shulman 1974). There are only few studies on carbohydrate allocation during the reproductive cycle of the fish. Accumulation of glycogen and glucose have been reported in the ovary of Oncorhynchus tschawytscha (Greene, 1926); Oncorhynchus nerka (Chang and Idler, 1960) and Clarius lazera (Yanni 1961). Belding (1934)

reported that female Salmo salar lose more carbohydrate from the muscle and viscera than do the males during maturation. In this species the glycogen content of the liver in a female spawner was found to be as little as 0.5 mg/gram while in male, it was recorded at 24.5 mg/gram (Fontaine and Hatey, 1953). Chang and Idler (1960) working on the same fish, further opined that the liver glycogen was preferentially depleted in the female fish. Studies on carbohydrate metabolism of the horse mackeral during the annual cycle by Morozova (cited from Shulman, 1974) revealed that though there were no clear-cut differences in the glycogen content of the muscle with respect to age or sex of the sample, there was a sharp decline in the glycogen content of the muscle during the spawning period. Shulman (1974) observed that the amount of glycogen in the muscle of salmonids was very small, irrespective of the degree of maturation. In Lampetra fluviatilis, Bentley and Follet (1965) reported that muscle glycogen falls from 245 mg% to 225 mg% at the time of spawning. The liver glycogen in the same fish was found to fall from 126 mg% to 14 mg%, when the fish was at the point of spawning death. One of the most important works, correlating carbohydrate depletion with the process of gametogenesis in male fish was that of Billard and Jalabert (1973). Though the source of depletion was not mentioned, the active deposition of

alfa and beta glycogen particles in the sertoli cells and spermatids, right from the beginning of spermatogenesis was reported in the guppy. The presence of this glycogen was believed to be related to the type of spermatogenesis occuring in the spermatogenic cysts and to facilitate the internal fertilization observed in the species, by acting as a source of energy. The values of total carbohydrate estimated in the present study in the various tissues of both the species of mullets are generally comparable with those observed in other species of teleosts. In the liver as well as in the muscle of the two species there was a gradual depletion of total carbohydrate with the advancement of maturity stages. However, in the testes of M. cephalus carbohydrate was found to increase only upto the third stage of maturity and in the case of L. parsia upto the second stage, after which there was a decrease. This might indicate that the depletion observed in the somatic tissue is not entirely for deposition in the gonad, but a part of the carbohydrate may be utilized for some other energy demanding activities such as spawning and migratory activity. Venugopalan (1962) working on O. striatus reported that when the ovary built up a glycogen reserve, the liver showed a depletion but during the post spawning stage, the liver glycogen showed an upward trend indicating a direct correlation. In the present study, such a direct correlation was not observed. This may be due to the fact

that the sperm during maturation does not require the storage of energy as in the case of the egg in the female, which stores energy for the subsequent utilization by the developing embryo. A comparison between the two species indicated that, while carbohydrates form a very insignificant part of reserve energy in M. cephalus, in L. parsia, it is of more importance.

During gonadal maturation and spawning, the lipid material of the fish, is utilised primarily for three purposes namely, (1) as an endogenous source of energy for sustaining the fish (as several of them are known to abstain from feeding during spawning) and for the increased muscular activity of the fish that have spawning migratory behaviour; (2) for the synthesis of generative materials (eggs and sperms), and yolk deposition and (3) for the synthesis of steroid hormones. The main sites of storage of lipids in the fish body are the subcutaneous connective tissue, liver, skeletal muscles, mesentry and interosseus tissue. Of these sites, the lipids from the liver seem to be the first to be mobilised during gonadal maturation in lean as well as fat fishes (Shulman, 1974). Idler and Bitners (1960) observed that in O. nerka 8% of muscle lipids is transferred to the ovary in the female, while only 0.5% is transferred to the testes in the male. Thurston and Newman (1962) calculated the degree of lipid depletion in this species and found that the lipids in the

white muscles drop from 9.7 to 1.8% while in the dark muscle from 27.4 to 6.8%. However, the degree of decrease was of similar proportion in both the tissues.

In certain fishes, as in the Atlantic herring, it is reported that there is an increase in the lipid content during gonad development although the fish does not feed intensely. It is suggested that the large number of organic acids produced during the transformation of proteins might be eventually converted to lipids resulting in the increase of this reserve material (Johnstone 1918). Although lipid in the liver and muscle undergoes marked changes during maturation and spawning, in the brain it remains at a constant level (Love, 1958). Marias and Erasmus (1977) reported that sexually mature specimens of *L. dumerilli* had considerably higher energy reserves during the off season than during the sexually active season. Pandey *et al.* (1976) working on *Heteropneustus fossilis* and Banerjee and Bagchi (1970) on *Labeo rohita* have indicated the same trend. The steady increase in the lipid content of the testes in *M. cephalus* from the first to the fourth stage, with the corresponding decline in the lipid content of the liver and the muscle observed at present study indicate that there is a positive correlation between the two processes. Though the results show that the muscle lipids tend to get depleted earlier than the liver lipids, the maximum drain has been from the lipid

reserve of the liver as it shows a sharp decline from 20.65% to 12.66%.

The increase in the lipid content of the liver from 17.71% in the first stage to 20.65% in the second stage, is indicative of an assimilatory phase of the fat depots during the preparatory stage; while, the subsequent decline could be due to its utilization for the various physiological processes related to maturation. The slight decrease in the lipid content of the gonad in the last two stages of maturation may be due to its utilization for the synthetic purposes. Further, as the sperms do not require the reserve food as in the case of the egg which stores food in the form of yolk, it is probable that once the formation of the generative cells is completed, the fish may not continue to divert its lipid resources to the testes. Lipid content of L. parsia is comparatively lesser than that of M. cephalus. The general trend of lipid depletion in L. parsia is the same as in M. cephalus. Here also the major amount of depletion is from the liver, from 6.22% in second stage to 2.93% in the sixth stage. However, the gonadal lipids in L. parsia do not show any sharp rise as in M. cephalus. The increase is gradual upto the third stage and becomes pronounced from the third to the fourth stage. The decline in the fifth and sixth stages is gradual. The results of the present study thus indicate that spermatogenesis

in both M. cephalus and L. parsia is an energy consuming process and draws upon the lipid reserves, which form the chief endogenous source of energy.

Cholesterol forms the most prominent sterol in the vertebrate cells. It is from this sterol that the common precursor for both andorgens and estrogens namely 'Pregnenolone' is synthesized. The estimate of the cholesterol content of the gonadal tissue thus gives an indication of the steroidogenic activity of the cells. The studies of Krum et al.(1964)in dogs revealed that testicular cholesterol formed an important precursor of steroidogenesis. Jayashree and Srinivasachar (1979) found that in Clarius batrachus, the lowest level of testicular cholesterol was observed during the peak spawning season, when the GSI was maximum, this was believed to be due to the utilization of the cholesterol reserves for the production of androgens. Callard and Ryan (1977) working on turtle have also arrived at the same hypothesis. However, Nayyar and Sundararaj (1970) working on Heteropneustus fossilis attributed the reduction in cholesterol content during spawning season to a reduction in the biosynthesising tissue (Leydig cells) in the testis during that time. Tepperman and Tepperman (1947) and Bartke (1971) working on rats, correlated the accumulation of cholesterol to an inhibition of androgen production in the testes. In Oncorhynchus nerka, Idler and Bitners

(1960) reported a decrease in the cholesterol content of both gonad and liver at the time of maximum sexual activity. However, they observed a slight increase in the muscle cholesterol during the same period (Idler and Bitners, 1958).

In the present study the cholesterol content of the three tissues namely, liver, muscle and gonad in M. cephalus, were found to get depleted with the advancement of maturation of the testes. While in liver there was a sharp decline from 2.17% in the first stage to 1.08% in the fourth stage, the muscle and gonad showed an initial increase and then a decrease. In the gonad the lowest values were recorded during the stages V and VI while in the muscle and liver, there was a substantial increase in the cholesterol values in these stages. This pattern of depletion and assimilation indicates that after a particular stage of maturity is reached (Stage IV in this species) the drainage of this triglyceride from its reserves stops, probably because, it is no longer utilized for steroid hormone synthesis. This cessation in hormone synthesis may be the starting point for the recycling of the cholesterol in its storage tissues. Unlike other energy reserves such as lipid and protein which tend to get accumulated in the gonad, with maturation, cholesterol tends to get depleted. This may perhaps be due to the fact that it does not primarily act as an energy giving substance but as a steroidogenic precursor which is actively

utilized in the early stages of spermatogenesis.

In L. parsia, the general trend of cholesterol depletion is similar to that of the lipids in the liver and muscle tissues. Unlike in M. cephalus, where the depletion of cholesterol is found up to the fourth stage followed by a redeposition in the last two stages, in L. parsia the depletion is found to continue upto the last stage of maturity without any redeposition. This may be because the lipid reserve of L. parsia is very small during the final maturity stage and cholesterol which forms only a very small part of this reserve, shows no appreciable change in its content. However, the gonadal tissue of L. parsia shows the same trend of cholesterol depletion as in M. cephalus. These observations thus reveal that the depletion of cholesterol is closely correlated with the synthetic activity of the gonad tissue. Since this triglyceride forms the precursor of the steroidal hormones, which play a pivotal role in the process of spermatogenesis, its depletion might be actually due to its utilization for the synthesis of these hormones.

Biochemical changes in serum

Under natural conditions the constituents of the blood serum in fishes are homeostatically maintained although subjected to minor fluctuations depending on

the physiological status of the organism. Such minor fluctuations, it is observed, would not upset the balance of the biochemical components in the blood upto a certain critical level. The fluctuations in the serum proteins during gonad maturation and spawning have been discussed in detail by Shulman (1974). In the horsemackerel the serum protein level was found to drop sharply during the prespawning period due to depletion for the synthesis of genital products, while in the goby, it is found to increase in the prespawning period due to active feeding. However, the serum protein level of the goby reached the lowest value, on the completion of maturation (Shulman, 1974)..The changes in the blood sugar with the activity of the fish was reported by Gray and Hall (1930). The studies of Barnhart (1969) in rainbow trout and Leach and Taylor (1976) in Fundulus heteroclitis indicated that there was no difference in the blood glucose levels between the sexes. Under stress condition however due to the effect of adrenalin, fishes are reported to show high levels of blood glucose (Menten, 1927). Studies on blood glucose levels of fishes subjected to starvation indicated that there was a decrease in most species, although the values were found to be fairly constant in species such as Cyprinus carpio and Carassius auratus (Varghese, 1983). Venugopalan (1962) correlated the fluctuations in the blood carbohydrate level with that of oogenesis in O. striatus.

Studies by Sulya (1960), Pickford et al.(1969) Takashima et al. (1972), Perrier et al. (1973), and Salfi et al. (1976) have shown that fish blood contains more lipid as compared to higher vertebrates. Under stress, hyperlipemic condition is often observed and this is attributed to the effect of plasma levels of adrenalin and noradrenalin. The annual cycle of serum gonadal steroids and serum lipids in striped mullet have been studied by Dindo and MacGregor (1981). Among the Indian workers Waseem and Siddiqui (1970) studied the biochemical and hematological parameters in the serum of Cirrhina mrigala and Labeo rohita; similar studies were also conducted by Sharma and Saxena (1979) in a few fresh water fishes. Jain et al. (1976) reported the effects of sexual maturation on the serum enzymes of Rita rita, while Dwivedi and Maria (1975) estimated the total serum protein in M. cephalus and Varghese (1983) reported on the protein, glucose and cholesterol content in the serum of Etroplus suratensis.

A general depletion of carbohydrate and protein levels in the blood serum of M. cephalus was observed in the advanced stages of maturity, during the present study. While the fluctuation in the carbohydrate level indicated an assimilatory phase from the first to the third stage followed by a phase of depletion up to the sixth stage, that of protein showed no distinct assimilatory phase in the initial stages of maturity. The protein level reached the minimum value in the fourth stage and thereafter steadily increased.

Anderson (1958) has reported heavy premigretory feeding in the case of the striped mullet. The increase in the carbohydrate content of the blood serum observed upto the third stage, in the present study, might perhaps be due to this active feeding behaviour.

The lipid content of the serum seems to rise and fall alternately between the first and the fourth stages of maturity, after which, there is a steady depletion upto the spent stage. Serum cholesterol on the other hand, showed only a downward trend from the first stage upto the fifth stage, followed by a sharp rise in the spent stage. This observation agrees with those made by Dindo and Mac Gregor (1981) in the striped mullet. The least values of serum cholesterol observed in the final stages of maturity might be due to the changes in cholesterol metabolism associated with the formation of steroidal hormones, as suggested by Love (1970).

Several of the chemical changes discussed above, in the somatic tissue during maturation can be duplicated by starvation of the fish (Love 1958; Yanni 1962; Creach and Cournede 1965). However, starvation, when it is not related to maturation, affect gonads adversely, for eg, in Salmo gairdneri severe starvation leads to resorption of eggs (Scott 1962); in Cyprinus carpio, testes lose weight during starvation (Creach and Cournede, 1965). In certain fishes like the salmon, a cessation in the feeding activity is

observed during the spawning migration. The depletion of the somatic biochemical components, observed during this period might not only be due to the drainage of these resources to the gonads but also due to their utilization for the maintenance of the fish during the nonfeeding period. Gulland (cited from Love, 1970) described the breakdown of the lining of the stomach in migratory Salmo salar by a process known as 'desquamaline catarrh'. Greene (1926) also made a similar observation in the same fish. Wootton (1979) suggests that the cessation of the feeding behaviour during the migratory run, observed in some fishes, might be a natural way of preventing cannibalism by the spawners of their own offsprings. Rae (1967a, 1967b) working on Gadus morhua and Milroy (1908) working on Clupea harengus observed that in these fishes, which feed intensely throughout the year, there was no significant depletion during maturation. Iles (1974) studying the growth pattern of fishes reported that the growth rate was greatly reduced after the fish attained maturity suggesting a diversion of the assimilated food for gonadal development. When the fish stopped feeding, there would be no assimilation of food, hence reserve food depots of the somatic tissues might be utilised both for maintenance and gonadal activity.

Brusle (1981a) reported that M. cephalus caught during the spawning run were all found to have empty stomachs indicating that the fish abstained from feeding during the spawning migration.

Besides the fluctuation in the biochemical components discussed above, the nucleic acids and vitamins also are reported to show some fluctuation during the period of gonadal growth and maturation (Love 1970). Among the nucleic acids, a substantial increase in the DNA content of the testes is only to be anticipated since DNA forms the major part of the sperm head and with advancement of maturity stages, the number of germ cells increase substantially. However, since DNA is not considered as a major source of energy during spermatogenesis (rather a major product of synthesis), the quantitative estimation of this nucleic acid has not been taken up.

In conclusion, the present study clearly brings out the variation in the levels of moisture, protein, carbohydrate and cholesterol in the various tissues of M. cephalus and L. parsia, during the different stages of maturity. In view of the observations made by the earlier workers, it may be concluded that these fluctuations may be partly due to the effect of starvation and partly due to the utilization of these parameters for the synthesis of the generative tissue, steroid hormones and other energy consuming activities of the fish in relation to maturation, spawning and migration. Further, the biochemical changes observed in the testes and the other body tissues (muscle, liver) during the maturation of the males, suggest that, like vitellogenesis in females, spermatogenesis also brings about considerable drain on the body resources and is an energy consuming process.

CHAPTER VIII

HISTOCHEMISTRY OF THE TESTICULAR CELLS DURING SPERMATOGENESIS

Histochemical methods are now a days extensively applied to understand the biochemical reaction and characterization of tissues, cells and organelles. Although it provides only qualitative information it has the advantage of studying the physiological activities or state of the cells and tissues without disturbing the normal structural organization. Realising this distinct advantage histochemical studies of the gonadal tissue of a number of fishes have been carried out by several workers like Krisnnamoorthy (1958) on Labeo fimbriatus, Mystus seengnala, and Boleophthalmus boddaerti; Venugopalan (1962) on Ophiocephalus striatus; Livni (1971) on Cyprinus carpio, Mugil capito and Tilapia aurea; Danto and Contini (1974) on Mugil chelo; Nagahama et al.(1976) on Carassius auratus; Sareen and Khullar (1977) on Discognathus lamta; Prasad and Gureya (1978) on Centrorhynchus corvi; Zhukinkis and Kim (1980) on Rutilus rutilus heckeli and Abramis brama and Verma et al. (1981) on Channa punctatus. However, all these studies are restricted to the histochemistry of the ovarian tissue.

In the case of the male gonad comparatively very few histochemical studies have been carried out. Of these, the preliminary histochemical observations on the testes of Gobius paganellus by Stanley et al. (1965), the histochemical studies during spermatogenesis of fresh water fishes such as Notopterus notopterus, Cirrhina mrigala, Labeo rohita, L. calabasu, L. gonius, Puntius chola, Tor tor, Ompok bimaculatus, Wallago attu and Mystus seengala by Upadhyay and Guraya (1973) and similar studies on the testes of Channa gachua by Shanbhag and Nadkarni (1979) are noteworthy.

In addition to the above, some of the workers investigated only certain aspects of gonadal histochemistry. Thus, Varo et al. (1979) studied the activity of acid phosphatase in the ovary of Carassius auratus gibelio and Cyprinus carpio, while Hart and Pontier (1979) dealt with the same aspect in the eggs of the zebra fish Brachydanio rerio. Similarly, the alkaline phosphatase activity in the oocytes of the blue gill, Lepomis macrochirus, was detected by Kugler et al. (1956), and that in the ovary of Labeo fimbriatus, Mystus seengnala and Boleophthalmus boddaerti during three different stages of ovarian development was studied by Krishnamoorthy (1959). The cyclical changes in lipids in the testes of Esox lucious was reported by Lofts and Marshall (1957). The seasonal variation in the lipid content of the gonad of Zoarces viviparous was worked out by Pekkarinen (1980).

Fostier et al. (1983) gave an excellent review of the gonadal steroids in teleosts. Steroidogenesis in the testicular tissue of Mugil cephalus was studied by Eckstein and Eylath (1968). Similar studies in the testes of a number of oviparous fishes were made by Hurk (1973). Colombo et al. (1978) described gonadal steroidogenesis in the sea bass, Dicentrarchus labrax and in the same year, Hoar and Nagahama reviewed the cellular sources of sex steroids in teleost gonads.

The effects of gonadotropin on the steroidogenic potential and spermatogenesis in Clarius batrachus received the attention of Rao et al. (1979). Recently Pekkarinen and Kristoiferson (1982) conducted histochemical studies of the steroid synthesising sites in Zoarces viviparous, while Hurk et al. (1982) described steroidogenesis in the gonads of rainbow trout fry. Changes in the free aminoacids in the different stages of the ovary of Labeo fimbriatus, Mystus seenghala and Boleophthalmus boddaerti were studied by Krishnamoorthy (1958). The role of glycogen during the formation and transport of spermatozoa of guppy in the male and female genital tracts were discussed by Billard and Jalabert (1973).

Since the reproduction in fishes is a highly complex energy consuming process involving the mobilisation of a large amount of nutritive materials which are utilized for the synthesis of new generative cells(gametes) and

hormones, and since no detailed study has been conducted on the histochemistry of proteins, carbohydrates, and lipids in the testicular tissue during the different stages of maturity and on their correlation with the synthetic activities of the various cell types in M. cephalus and L. parsia, an attempt is made here to demonstrate histochemically the changes in the major biochemical constituents in these fishes during spermatogenesis.

Histochemical tests and their interpretation

Proteins, carbohydrates and lipids are demonstrated by chromogenic agents which selectively take up the stain so as to enable their identity. The specific tests for each type of protein, carbohydrate and lipid are given in the table 15 and the procedures adopted for blocking the reactive groups are given in table 16. The intensity of staining in each case is marked by arbitrary symbols, - (no reaction); + (weak); ++ (moderate); +++ (strong); and ++++ (highly intense). The absence of a cell type in a particular stage is indicated by (*). The tests are performed in a specific sequence from the more general ones to the specific types.

Protein: Mercuric bromophenol blue test is used as a general test for proteins since testicular tissue is found to respond to this test very distinctly. Adequate

TABLE - 15. Histochemical tests and the corresponding reactive groups.

	Tests	Reactive groups
PROTEINS	Mercuric Bromophenol Blue (MBB)	Proteins (deep blue)
	Aqueous Bromophenol Blue (ABB)	Basic proteins (blue)
	Ninhydrin - Schiff	Amino groups (pink)
	Toluidine Blue	Acidic groups(red/purple)
	Ferric-Ferricyanide	-SH groups (blue)
	Performic acid - Alcian Blue (PBAB)	SS groups (greenish blue)
	Millon's test	Tyrosyl group (red/pink)
	P-Dimethylamino-benzaldehyde (DMAB)	Tryptophan (deep blue)
CARBOHYDRATES	Schiff's reagent	Free aldehydes (magenta)
	Periodic Acid - Schiff (PAS)	1,2 glycol groups (magenta or purple)
	Best's carmine	Glycogen (red)
	Toluidine Blue at pH 1.99) pH 3.09) pH 4.19)	Sulphated - Acid mucopolysaccharides (AMP) (blue)
	pH 7.0	carboxylated acid mucopolysaccharides (blue)
LIPIDS	Sudan Black B	Lipids (bluish black)
	1% Nile Blue at 60°C	Neutral lipids (red) Acidic lipids (blue)
	Nile Blue with sulphuric acid	Phospholipids (blue)
	Oil red O'	Neutral lipids (blue)
	Romieu's test	Cholesterol (pale violet turns green)

TABLE - 16. Blocking reactions and extraction procedures of specific reactive groups

	Methods	Reactive groups
1.	Deamination	Removal of amino groups
2.	Methylation	Removal of acidic groups
3.	Mercaptide	Blocking of -SH groups
4.	Iodination	Blocking of -OH groups
5.	40% Formaldehyde	Blocking of tryptophan
6.	Acetylation	Blocking of 1,2 glycols
7.	Deacetylation	Reversal of acetylation
8.	Chloroform - Methanol extraction	Removal of lipids
9.	Pyridine extraction	Removal of phospholipids
10.	Taka diastase treatment	Removal of glycogen

histochemical procedures are not available for all the aminoacids and hence the determination of the specific type of proteins in the tissue sections are limited to a small number of available procedures that are found suitable. The specificity of each test for a particular reactive group is confirmed by staining control slides that are subjected to blocking procedures, that blocked the specific reactive groups.

Carbohydrates: Though Periodic Acid-Schiff (PAS) is commonly used as a general test for carbohydrates, all biomolecules with 1,2 glycol groups are found to respond to this test and hence a number of control sections are employed with different blocking procedures to identify the specific types of 1,2 glycols present.

Lipids: Histochemistry of lipid depends on the solubility of the dye in the lipid to be tested. When tissue sections are exposed to the staining solutions, the dye dissolves into the tissue lipid perferentially. Control sections are exposed to lipid extraction methods before staining. A comparison between stained sections of both extracted and unextracted material indicates the type of lipids present. Sudan black B is used as the general stain to detect lipids. It stains bound and free lipids in shades of bluish black.

RESULTS

The results of the histochemical tests are indicated in tables 17 and 18. A consolidated summary of the results are given in tables 19 and 20.

A. M. cephalus

1. Protein:

1.1. Connective tissue: The connective tissue has a very low content of general proteins in the first three stages of maturity. It attains a peak level in the fourth and fifth stages and thereafter decreases gradually in the spent stage. Basic proteins also show more or less a similar trend of distribution. The tests for amino groups and acidic groups elicited only weak responses in the first three stages. In the fourth stage however, there is a sharp rise in the staining intensity of amino groups while only a moderate increase in the staining intensity of the acidic groups. The staining intensity of the amino groups gradually decreased and reached the minimum intensity in the sixth stage while that of the acidic groups remained the same up to the last stage of maturity. Although the test for - SH groups indicated their presence from the first stage onwards, it was moderately positive only in the fourth stage.

The SS groups also indicated their presence in all stages except the spent stage with a moderately intense staining in the third stage. Traces of tyrosyl and tryptophanyl groups could be detected in all the stages with a slightly higher concentration in the fourth stage.

1.2. Interstitial cells: Proteins in general are detected in the interstitial cells in all the stages, with maximum intensity in the fourth stage, moderate intensity in the first, second, third and fifth stages and least intensity in the sixth stage. Basic proteins are moderately positive in all stages except the spent stage. The presence of amino groups could not be detected in the first stage, however, they are faintly positive in the second, third, fifth and sixth stages with moderately intense staining in the fourth stage. Acidic groups also gave a faintly positive response in all the stages except the first and second. -SH and SS groups were all faintly positive up to the fifth stage but could not be detected in the sixth stage. Tyrosine and tryptophan showed faint staining in the first three stages and moderately intense staining in the fourth stage. Tyrosine could not be detected in the last two stages, while traces of tryptophan could be seen in the last two stages also.

1.3. Spermatogonia: The spermatogonial cells are moderately positive to the test for general proteins in all the stages of maturity. A weak response to basic proteins is seen in the first two stages while in the

last four stages they seem to be moderately positive. Amino and acidic groups appear to be moderately intense in the third, fourth and fifth stages but faintly positive in the sixth stage. Traces of tyrosine could be detected in the first stage alone, -SH groups in the first two stages and tryptophan in the first three stages.

1.4. Spermatocytes: Spermatocytes make their appearance only from the second stage onwards. The general proteins show a gradual increase in staining intensity from the second to the fourth stage and there after decreases in the sixth stage. Basic proteins can be faintly detected in the second stage. In the third, fourth and fifth stages they are moderately positive but become weakly positive in the sixth stage. Amino groups are faintly detected in all the stages from the second to the sixth. Acidic groups are detected only in the fourth and fifth stages. Tests for -SH groups are weakly positive in second and fifth stages but moderately positive in the third and fourth stages. SS groups are detected only in the fourth and fifth stages. Traces of tryptophan are observed from the third to the sixth stage.

1.5. Spermatids: Spermatids are observed from the third stage onwards. General and basic proteins are present in high concentrations in the third, fourth and fifth stages as indicated by their strong staining reactions. In the spent stage however, they are only moderately present. Traces of amino groups are present

in the third, fourth and fifth stages while that of acidic groups are detected only in the fourth and fifth stages. A moderately positive reaction for the -SH groups is observed in the third stage. The histochemical tests for SS groups, tyrosine and tryptophan, however, are found to be negative in the different stages.

1.6. Spermatozoa: Spermatozoa can be seen only in the fourth, fifth and sixth stages. In all these stages, they are strongly positive to general proteins. The spermatozoa of the fourth and fifth stages are intensely positive to basic proteins while the degenerating sperms of the spent stage are only strongly positive to basic proteins. Traces of amino groups can be seen in the fourth and fifth stages. No other tests for proteins seem to elicit any response.

2. Carbohydrate:

2.1. Connective tissue: Free aldehydes are weakly detected in the connective tissue in the first and second stages. The presence of 1,2 glycols is seen in all the stages with the intensity of staining being highest in the second and third stages, moderate in the first and weak in the last three stages. Glycogen is detected in the first four stages. It is present in traces in the first and fourth stages but moderately intense in the second and third stages. Sulphated acid mucopolysaccharides are present in all the

stages except the spent stage. It is moderately positive in the second stage and weakly positive in the other stages.

2.2. Interstitial cells: The interstitial cells of the testes do not indicate the presence of free aldehydes. They are moderately positive to 1,2 glycols in the second stage but only weakly positive in the rest of the stages. Traces of glycogen are detected in the first, third, fourth and fifth stages. Sulphated acid mucopolysaccharides are present in the first five stages, with moderately intense staining with the second stage.

2.3. Spermatogonia: The spermatogonial cells respond to tests for carbohydrates only in the first four stages. Free aldehydes are not detected in any of the stages. A moderately intense staining is seen in the case of 1,2 glycols and glycogen in the second stage while only traces of these groups are present in the other stages. Traces of sulphated acid mucopolysaccharides are also seen in the second and third stages.

2.4. Spermatocyte: Spermatocytes respond to tests for carbohydrates only in the second, third, fourth and fifth stages. Free aldehydes and glycogen are not detected in any of the stages. A moderately intense positive reaction is seen to 1,2 glycols in all the stages except the second where it is present only in traces. Sulphated acid mucopolysaccharides are weakly detected in all the stages from the second to the fifth.

2.5. Spermatid: These cells appearing from the third stage respond only to the PAS test, indicating the presence of 1,2 glycols in the third, fourth and fifth stages.

2.6. Sperm: These mature gametes observed from the fourth stage onwards only give weak response to 1,2 glycols in the fourth and fifth stages.

3. Lipid:

3.1. Connective tissue: The cells of the connective tissue respond to all the tests for lipids in all the stages. General lipids are detected in traces in the first and second stages while they are moderately positive in the third, fourth, fifth and sixth stages. Neutral and acidic lipids are only weakly positive in all the stages. Phospholipids are detected in traces in all the stages, except the second. Similarly cholesterol is observed in traces in all the stages except the third. Phospholipids are moderately positive in the second stage and the cholesterol in the third stage.

3.2. Interstitial cells: General lipids are only weakly positive in the first, fifth and sixth stages in the interstitial cells while they are moderately positive in the other stages. Neutral lipids are moderately positive in the second stage and weakly positive in rest of the

stages. Acidic and phospholipids are detected in traces in all the stages. Cholesterol is weakly positive in the first stage, moderately positive in the second and strongly positive in the third stage. In the fourth and fifth stages it can be detected in traces but not in the last stage.

3.3. Spermatogonia: The spermatogonial cells seen in the first four stages alone, respond to tests for lipids. Only weakly positive reactions are seen for general lipids, phospholipids, neutral and acidic lipids in all the four stages. Cholesterol is not detected.

3.4. Spermatocyte: Spermatocytes show traces of general lipids and phospholipids in the second, third, fourth and fifth stages. The residual spermatocytes of the sixth stage do not respond to any of the tests for lipids.

3.5. Spermatids: From the third stage onwards cysts of spermatids that are weakly positive to general lipids and phospholipids can be observed. These cells do not respond to any of the tests for lipids in the sixth stage.

3.6. Spermatozoa: As in spermatocytes and spermatids, only general lipids and phospholipids are observed in the spermatozoa, which are seen from the fourth stage onwards. The degenerating spermatozoa of

the sixth stage do not respond to any of the tests for lipids.

B. L. parsia

1. Protein:

1.1. Connective tissue: The protein level of connective tissue seems to be quite high. The general proteins show an increase from a moderately positive reaction in the first two stages to a strongly positive reaction in the third, fourth and fifth stages, followed by a moderately positive reaction in the spent stage. Basic proteins also follow the same pattern up to the fifth stage. In the sixth stage it is only weakly positive. Amino groups are detected in traces in all the stages except the fifth where it is moderately positive. Acidic and - SH groups are weakly positive in the first three stages and moderately positive in the fourth and fifth stages. In the spent stage, acidic groups are moderately positive while -SH groups are weakly positive. The staining intensity of the SS groups is moderately positive in the third and fourth stages and weakly positive in the rest of the stages. Tyrosine is detected in traces in all the stages except the fourth stage where it is moderately positive. Tryptophan is moderately positive in the second stage and faintly positive in all the other stages.

1.2. Interstitial cells: The interstitial cells of L. parsia are positive to tests for general and basic proteins in all the stages of maturity. They are moderately positive in the first, second, fourth and fifth stages, strongly positive in the third stage and weakly positive in the last stage. Tests for amino groups elicit only a weakly positive response in all the stages. Acidic groups are not detected in the sixth stage but they are moderately positive in the fourth stage and weakly positive in all the other stages. A moderately positive response to -SH groups is noticed in the fourth stage while there is only a weak response in the rest of the stages. SS groups are not detected in the third and fourth stages but they are present in traces in the first, second fifth and sixth stages. Tyrosine is present in traces in the first, second, third and fifth stages and tryptohan in all the stages except the fourth.

1.3. Spermatogonia: These cells are moderately positive to general proteins in all the stages of maturity. Basic protein content of the spermatogonial cells is very low in the first two and fifth and sixth stages while it is moderately positive in the third and fourth stages. Amino groups are detected in traces in all the stages except the fifth stage where it is moderately positive. Acidic groups are weakly detected in the second and third stages but they are moderately positive in the fifth stage. Traces of -SH groups are detected in the first three stages while those of SS groups and tyrosine are detected only in the first and

second stages. Tryptophan is weakly positive in the first stage, moderate in the second, but not detected in the rest of the stages.

1.4. Spermatocyte: General and basic proteins follow almost the same pattern of fluctuation in the spermatocytes as those observed for the spermatogonial cells. Spermatocytes show a moderately positive reaction to general and basic proteins, up to the fifth stage followed by a weakly positive response in the last stage. Amino groups are detected in traces in the second, third and fourth stages, followed by a moderately positive reaction in the fifth stage. A weakly positive response to acidic groups is seen only in the fourth and fifth stages. -SH groups are found to increase in staining intensity from traces in the second stage to moderately positive condition in the third and fourth stages, followed by a weakly positive condition in the fifth stage. -S-S- groups are not detected in any of the stages. Tyrosine is only weakly positive in the second and fourth stages. Traces of tryptophan are detected in the second, third, fourth and fifth stages.

1.5. Spermatids: General proteins give a strongly positive response in the fourth stage, moderately positive response in the third and fifth stages and a weak response in the sixth stage. Basic proteins are strongly positive from the third to the fifth stages but moderately positive in the spent stage. Amino group is detected only in traces

in the third, fourth and fifth stages. Acidic group is moderately positive in the fourth stage but weakly positive in the fifth stage. -SH group is found in traces only in the third stage. SS group, tyrosine and tryptophan are not detected in any of the stages.

1.6. Spermatozoa: General and basic proteins are intensely positive in fourth and fifth stages and strongly positive in the sixth stage. Amino group is only weakly detected in the fourth and fifth stages. Acidic group, -SH group, tyrosine and tryptophan are not detected in any of the stages.

2. Carbohydrate:

2.1. Connective tissue: A weak response to free aldehydes is seen in all the stages except the fourth and sixth. 1,2 glycols are strongly positive in the second stage, weakly positive in the fifth stage and moderately positive in the rest of the stages. Glycogen is strongly positive in the second stage, moderately positive in the third stage and weakly positive in the rest of the stages except the sixth where it is not detected. Sulphated acid mucopolysaccharides are detected in all the stages with moderately positive reaction in the first and third stages and weak response in the rest of the stages.

2.2. Interstitial cells: Weakly positive response to free aldehydes is seen in the third and fifth stages.

1,2 glycols are moderately positive up to the fourth stage after which they are seen in traces in the fifth stage. Traces of glycogen are observed in all the stages up to the fourth. It is absent in the fifth but reappears in the sixth stage. A weak response to sulphated acid mucopolysaccharides is seen in all the stages except the sixth.

2.3. Spermatogonia: Free aldehydes are not detected. 1,2 glycols are moderately positive in the second stage and weakly positive in the third and fifth stages. Glycogen and sulphated acid mucopolysaccharides are detected only in the first four stages. Glycogen is weakly positive in the first stage, moderately positive in the second and weakly positive in the third and fourth. Sulphated acid mucopolysaccharide is weakly positive in the first but moderately positive in the next three stages.

2.4. Spermatocyte: As in the case of spermatogonial cells, free aldehydes are not detected. 1,2 glycols are present in traces in the third stage. Traces of sulphated acid mucopolysaccharides are found in the second and third stages but in moderate intensities in the fourth and fifth stages.

2.5. Spermatid: These cells respond only to the test for sulphated acid mucopolysaccharides which are detected in traces in the third, fourth and fifth stages.

2.6. Spermatozoa: Like spermatids, the spermatozoa also respond only to the test for sulphated acid

mucopolysaccharides. These cells show a weak response to this group in the fourth and fifth stages.

3. Lipid:

3.1. Connective tissue: The cells of the connective tissue give a weak response to general lipids in the first five stages but a moderately positive response in the sixth stage. Tests for acidic lipids, phospholipids, neutral lipids and cholesterol elicit a weak response in all the stages.

3.2. Interstitial cells: Interstitial cells contain the maximum amount of lipids as compared to any of the cell types in the testis. They are moderately positive to general lipids in all the stages except the sixth stage. The test for neutral and acidic lipids elicit only a weak response in the first five stages. Phospholipids are moderately positive in the first four stages but weakly positive from the second to the fifth stage. Cholesterol is detected in all the stages with a strongly positive response seen in the third stage and a weak response in the rest of the stages.

3.3. Spermatogonia: Very little lipid is detected in these cells. A weak response to test for general lipids is seen in all the stages of maturity. The test for neutral and acidic lipids show a weakly positive

response in the first four stages. Phospholipids are detected in traces in the third, fourth and fifth stages.

3.4. Spermatocyte: General lipids are detected in traces in the second, third and fourth stages. Similarly, a weak positive reaction to acidic lipids is also seen in the second, third and fourth stages. Phospholipids can be detected only in the third, fourth and fifth stages while neutral lipids and cholesterol are not detected.

3.5. Spermatid: A weak response to general lipids is seen in the third and fourth stages. Tests for phospholipids elicit a moderately positive response in the fourth stage and a weakly positive response in the third, fifth and sixth stages.

3.6. Spermatozoa: Traces of general lipids can be detected in the fourth stage while phospholipids are detected in fourth, fifth and sixth stages. No other tests for lipids elicit any response.

DISCUSSION

In the chapter on the biochemical analysis, the translocation of major nutrients from the soma to the testes during the different stages of maturation was discussed.

It was further shown that as in ovarian maturation of fishes, spermatogenesis was also a dynamic process and involved subtle changes in the body constitution with the advancement of maturation. The present histochemical studies of the testicular cells during maturation helps in providing qualitative information of the biochemical changes.

protein: Proteins form the building blocks of any body tissue. The amino acids derived from the breakdown of specific types of proteins form the precursors for the synthesis of the generative cells (sperms) and the enzymes that catalyse all the related synthetic and metabolic activities.

The response to the tests for proteins was more or less similar in M. cephalus and L. parsia. The high intensity of staining with mercuric bromophenol blue and aqueous bromophenol blue noticed in most of the cells in the third, fourth and fifth stages of maturity indicated the increase in the proteinaceous material in the testicular tissue during the peak of spermatogenesis. The histochemical characterisation of the proteins further revealed that these proteins responded to tests for acidic groups, amino groups, -SH groups, SS groups, tyrosyl and tryptophanyl groups.

Though the cells of the connective tissue and the interstitial tissue were positive to all the tests for

proteins in the final stages of maturity, the intensity of staining for the specific reactive groups was much less in the early stages and in the spent stage, indicating that the protein content of these cells undergo fluctuation with respect to the maturity stage of the gonad. A similar condition was observed by Craig-Bennet (1930) in Gasterosteus. It is speculated that these cells act as the specific sites for the storage and synthesis of the vital proteins and enzymes, required for biosynthetic activities such as steroidogenesis.

The spermatogonial cells at different stages showed variation in their staining properties. While the quiescent spermatogonia of the first and second stages gave very weak reaction to the tests for basic proteins, amino groups, -SH groups, tyrosine and tryptophan, the actively developing spermatogonial cells of the third, fourth and fifth stages, that were in the synthetic phase of the cell cycle prior to cell division, were found to be moderately positive to tests for basic proteins. Amino groups and acid groups were also found to be moderately positive in the fourth and fifth stages. These differences in the staining properties between the spermatogonial cells of the early stages of maturity as compared to those of the late stages of maturity may be due to the assimilation of aminoacids such as glutamine, aspartic acid, and glycine that form the precursors for the synthesis of new DNA and RNA molecules in the latter.

The spermatocytes as in the case of the spermatogonial cells also show varying intensities of staining for proteins in the different stages of maturity. This might again be due to the variation in the synthetic activity of the cell. The highest intensity of staining for proteins and the maximum number of reactive groups were noticed in the third, fourth and fifth stages. The proteins detected were basic proteins, amino groups, acidic groups, -SH groups and SS groups. Korzhenko (1966) has reported that in Oncorhynchus keta there is an accumulation of amino acids having high nitrogen content (arginine, histidine, leucine, lysine, isoleucine and valine) at the expense of nitrogen poor compounds (serine, proline and glycine). It was suggested that this phenomenon was in some way related to the synthesis of nucleic acids. The presence of -SH containing amino acids (cysteine or methionine), SS containing amino acids (cystine) and basic aminoacids (lysine, arginine or hydroxylysine) in the spermatocytes of M. cephalus and L. parsia indicate the same possibility.

The spermatids and sperms of M. cephalus and L. parsia are predominantly positive to general and basic proteins. The staining intensities of these cells to aqueous bromophenol blue is significantly greater than that of any other cell type in the testis. This may be due to the presence of large amounts of arginine which form the major component of the fish sperm protamines.

Protamines were identified as special types of nucleoproteins by Miescher (1874) in salmon (cited from Delange and Smith, 1979). Arginine forms about two thirds of the residues in a protamine molecule. Only four to eight other amino acids such as proline, alanine, serine, threonine, glycine, valine, isoleucine and tyrosine all of which are neutral amino acids have been detected in addition to arginine in the protamines sequenced so far (Delange and Smith, 1979). However, neutral amino acids such as leucine, phenylalanine, tryptophan, cysteine and aspargine and acidic aminoacids have not been found in any of the protamines studied (Delange and Smith, 1979).

During spermiogenesis, synthesis of protamines takes place in the cytoplasm of the spermatids, from where they are transported to the nucleus where they replace histones and remain complexed with DNA (Delange and Smith, 1979). Towards the end of spermiogenesis, the nuclear histones are completely replaced by protamines. The replacement of histones by protamines is significant because these proteins are known to bind to DNA in a manner by which DNA synthesis is completely repressed and the chromatin material becomes highly condensed and reduced in volume. This is an important phenomenon since unlike other cell types the sperms do not have to participate in protein synthesis. Further the condensation of the chromatin material enables the sperm head to acquire extreme

compactness making their movement easier (Berrill and Karp, 1978). Histones would have been too bulky to accomplish this task (Delange and Smith, 1979).

Histones are known to contain traces of tryptophan. The presence of tryptophan together with acidic and basic proteins in the spermatogonia and spermatocytes suggest that histones form the major nuclear proteins in these cells, while the intense positive reaction to basic proteins and the absence of acidic groups and tryptophan, noticed in the spermatozoa suggest that protamines are synthesised in the final stages of spermatogenesis in M. cephalus and L. parsia. The high degree of condensation of the chromosome material observed in the electron micrographs of the late spermatids and spermatozoa (Plate XVII, Fig. 5; Plate XVIII, Fig. 1; Plate XXV, Figs. 4 and 5) in M. cephalus and L. parsia also confirm the presence of these proteins.

Carbohydrate: The literature on the histochemical characterisation of the carbohydrates of fish gonad is very limited. Most of the investigations on the histochemical characterisation of carbohydrates are restricted to the female gonad. Lindberg (1945) and Monne and Slautterback (1951) made significant studies in the sea urchin, while Fitch and Merrick (1958) made similar studies in frog Rana pipiens. In fishes, the works of Daniel (1947) in salmon, Kugler et al. (1956) in Lepomis macrochirus and

Venugopalan (1962) in Ophiocephalus striatus are noteworthy.

Meischer (cited from Giese, 1973) found that about 60% of the solids in the fish spermatozoa was made up of nucleic acids, 35% proteins and 5% lipids, salts and carbohydrates. The most significant study on the variation in carbohydrates in the male gonad of fishes, is made by Billard and Jalabert (1973) in guppy. In this fish which has internal fertilization, they report that both alpha and beta glycogen particles are present in the sertoli cells and spermatids during spermatogenesis. The glycogen in the sperm is reported to be localised around the mitochondria of the midpiece. It is suggested that the type of glycogen present may be related to the type of spermiogenesis occuring in the cysts, which prevents the release of polysaccharides in the testicular fluid. The decrease in the glycogen reserve observed among stored spermatozoa are interpreted to be due to the depletion of glycogen reserve while swimming. It is therefore considered as an endogenous source of energy (Billard and Jalabert, 1973).

The response to the tests for carbohydrates have been more or less similar in M. cephalus and L. parsia in all the stages of maturity. Traces of free aldehydes are detected in the connective tissue of the first and second stages of the testes in M. cephalus and L. parsia while it is absent in all the other stages. The positive reaction to PAS test in the connective tissue of all stages

indicates that substances of high molecular weight containing 1,2 glycol groups such as polysaccharides, hyaluronic acid, mucoproteins or mucins and glycogen may be present. Monosaccharides, sugars and cerebrosides would not be present as they would have been removed by aqueous fixatives (Pearse, 1968). PAS tests conducted after blocking reactions such as acetylation and deacetylation show that the PAS positive substances mostly consisted of polysaccharides. The positive reaction after chloroform - methanol extraction further confirms this. The intensity of the staining reaction reveals that there is a slight increase in the concentration of carbohydrates from stage I to stage III in M. cephalus followed by a decrease, while in L. parsia there is only a mild fluctuation from a strongly positive response in the second stage to a moderately positive condition in the subsequent stages. This may be because during the early stages, the carbohydrates mobilised from other tissues might be partly laid down in the connective tissue as reserve material, while in the the later stages these might be metabolised for providing energy for the biosynthetic activities of the testes. The staining intensity of glycogen is found to be slightly high in the connective tissue in the second and third stages as compared to the first and the last three stages showing a clear symptom of depletion. Sulphated acid mucopolysaccharides also follow a similar trend of depletion in M. cephalus while in L. parsia they show only mild fluctuation.

The presence of sulphated acid mucopolysaccharides is significant due to their unique property of readily forming reversible linkages with other compounds. This property enables them to bind large amounts of salts and water (Jackson, 1964). It is therefore suggested that these molecules play an important role in regulating the diffusion mechanism and the inorganic ion metabolism during spermiation in the testes.

The positive reaction to sulfated acid mucopolysaccharides also suggest the presence of chondroitin sulphates, which in addition to controlling the concentration and ionic strength of the cell wall inhibit blood clotting and offer resistance to testicular hyaluronidase activity (Jackson, 1964).

The interstitial cells of M. cephalus and L. parsia show a positive reaction to the PAS test. Here too the intensity of staining is minimum or nil in the spent stage. Glycogen and sulfated acid mucopolysaccharides are present only in traces in these cells in both the species.

The spermatogonial cells of M. cephalus and L. parsia give the same response to the PAS and glycogen tests while the spermatocytes only respond to PAS and sulfated acid mucopolysaccharides. The spermatids and sperms of M. cephalus do not indicate the presence of sulfated acid mucopolysaccharides or glycogen but those of L. parsia

show positive reaction to sulfated acid mucopolysaccharides.

In general, the histochemical analysis of carbohydrates in the testicular tissue during different maturity stages confirm the results of the biochemical studies presented earlier, in that the carbohydrates as compared to proteins are present in very small quantities in the testicular tissue. This may be because, the major role of carbohydrates is to provide energy and in fishes, incorporation of such energy rich compounds in the gonads is usually noticed in the females during vitellogenesis of the oocytes. Since the teleost sperm has a very short life span there is no need for the accumulation of large carbohydrate reserves in the cytoplasm.

Lipids: The histochemistry of the testicular tissue of M. cephalus and L. parsia reveals that in all the stages of maturity lipids are detected mainly in the connective tissue and the interstitial cells.

The major role of the testicular lipid is that it forms the precursors for the synthesis of steroid hormones. A number of workers have investigated the process of steroidogenesis in different groups of fishes. A review of these studies has been given by Fostier et al. (1983). In M. cephalus, Eckstein and Fylath (1968) studied steroidogenesis in the testicular tissue and Azoury and

Eckstein (1980) studied the same during oogenesis. In recent times the presence of 3β hydroxysteroid dehydrogenase, 11β hydroxysteroid dehydrogenane and 17β hydroxysteroid dehydrogenase , have been taken as the criteria for the steroidogenic activity of the cells (Hurk et al., 1978b). As per these histoenzymological observations, interstitial cells (Leydig cells) are recognised as the main sites for the synthesis of steroids. This finding supports the earlier hypothesis put forward by Barrington (1968) which recognises interstitial cells as the sites of secretion of male steroid harmones in all the major vertebrate groups.

The presence of Sudanophilic substances in the interstitial and connective tissue cells of the testis of M. cephalus and L. parsia together with the specific positive reaction of these cells to the test for cholesterol suggests the possible steroidogenic activity of these cells. Though similar substances are observed in the ovary of fishes, Lofts and Bern (1972) have opined that presence of Sundanophilic lipid droplets containing triglyserides, cholesterol or phospholipids are not specific and should be considered cautiously, it is felt that as far as testes is concerned, the presence of these substances must be indicative of steroidogenesis, since unlike in oocytes, large amounts of nutritive substances need not be accumulated in the sperms since they have a very short life span.

In the present study, cholesterol is found in the somatic cells of the testes in almost all the stages with the intensity of staining being greater in the first three stages as compared to the last three in both the species. This may be due to the utilization of this triglyceride for the synthesis of pregnenolone and progesterone which form the precursors for the synthesis of male steroid hormones such as dehydroepiandrosterone, androstenedione and testosterone, during the peak of spermatogenic activity in the final stages of maturation. Eckstein and Eylath (1968) have identified pregnenolone and progesterone in the testicular tissue of M. cephalus during spermiation.

The characterisation of the testicular lipids in M. cephalus and L. parsia revealed that, apart from cholesterol they also contained neutral and phospholipids as shown by the positivity to Oil red 'O' and Nile blue sulfate tests. However, these were present only in traces. It has already been established that neutral lipids play an important role as energy reserves during embryonic development (Giese, 1973). The presence of these lipids in the male gonad perhaps suggest their function as energy reserves for the synthesis and maintenance of spermatozoa.

In contrast to the test for neutral lipids, that was positive only in the somatic cells of the testes, the

test for phospholipids yielded a positive reaction in all the cell types especially in the fourth and fifth stages of maturity. This is significant because in addition to supplying metabolic energy to the sperms during their short span of motile life, phospholipids are also concerned with structural integration and membrane formation of the cells.

The results of the histochemical studies are in agreement with that of the biochemical changes discussed earlier. Though sperms are known to contribute only genetic material to the zygote while eggs supply both genetic material as well as nutrients for the development of the embryo, it is seen that the synthesis of the sperms and the steroid hormones is an energy consuming process that accounts for the translocation of the biochemical components from the soma to the testes.

CHAPTER IX

PRELIMINARY STUDIES ON THE CRYOPRESERVATION OF MILT OF *MUGIL CEPHALUS* AND *LIZA PARSIA*

The increasing awareness of the need for genetic conservation of aquatic organisms that are subjected to exploitation through capture and culture means, hazards of pollution and environmental deterioration, has attracted considerable attention on the studies on cryobiology and cryopreservation of the gametes. Besides, the technology of cryopreservation of gametes is found to facilitate hybridization and artificial propagation of strains or species that are geographically seperated or spawn at different times or seasons. Further it also helps to economise the hatchery establishment and operation by reducing the maintenance of brood stocks. Recognising the significant role of cryopreservation of gametes in the breeding programmes and in view of the long term need for preserving the natural genetic variability of the commercially exploited species, the FAO Technical Conference on Aquaculture held in Japan in 1976 as well as the Nordic Symposium on 'Gene Banks' held in Helsinki in 1978 recommended the establishment of gene pools and egg/sperm banks through appropriate methods of preservation of fish eggs and milt.

Although the storage of the fish milt for longer duration at low temperature was reported as early as 1914, the techniques of cryopreservation of fish gametes generally followed those developed for mammals. Introducing suitable modifications in this technology, considerable progress in the cryobiology of fish gametes, particularly of milt, has been made since 1970. Several reviews are now available on the different aspects of the subject by Blaxter (1969), Shehadeh (1975), Horton and Ott (1976), Harvey and Hoar (1979), Billard (1980), Scott and Baynes (1980), Pullin and Kuo (1981), Sunderaraj (1981), Withler (1981), Stoss and Donaldson (1982) and Stoss (1983). Most of the investigations pertain to salmonid fishes and to those from freshwaters; studies on marine fishes are comparatively few. Among mullets, the only species investigated so far is Mugil cephalus. Hwang et al. (1972) studied the effect of a series of temperature ranges from 24°C to - 20°C on M. cephalus sperms. This work was followed later by those of Chao and his team (Chao et al., 1975 and Chao, 1982) using different extender media.

Studies on cryopreservation of fish gametes from India are limited to the experiments carried out by Bhowmick and Bagchi (1971) on the preservation of sperms of rohu Labeo rohita and common carp, Cyprinus carpio. The milt of these species, collected at room temperature

(22-33°C) were mixed with diluents such as egg yolk-citrate (M/15 and M/7), Sodium citrate (M/7), phosphate buffer, Holtfreter's solution and frog Ringer solution at temperatures of 0 to 5°C under refrigeration. It was observed that of the different diluents used Holtfreter's solution containing 1% glycerine gave encouraging results. It is reported that short term preservation of carp sperm has been achieved at the Central Inland Fisheries Research Institute and further studies in this direction in fresh water fishes are in progress at the Institute in collaboration with the University of Delhi (Kuldip Kumar, personal communication).

There is practically no information on the cryopreservation of marine fishes in India. In view of this and the great potentials for aquaculture of mullets in the country, an attempt is made to study certain aspects of cryobiology of sperms of M. cephalus and L. parsia and cryopreservation of milt of the species in different cryoprotectants and extender media. The results obtained are presented and discussed. A brief review of the progress made in the field of cryobiology and cryopreservation of fish gametes is also given.

Cryobiology and Cryopreservation of fish gametes

Although the studies of Smith and Quistorff (1943) and Okada et al. (1956) showed that the sperms obtained

from the testes after 1½ to 5 hours of the death of the fish remained viable, the capacity of the sperms to fertilize the eggs was found to decrease rapidly within 18 hours of post-mortem storage in most of the cases. In this context, the observation reported earlier by Brofeldt, 1914 (cited from Buyukhatipoglu and Holtz, 1978) that the trout and salmon sperm could survive longer at low temperatures, attracted the attention of Scheuring (1925), Smith and Quistorff (1943), Barret (1951), Henderson and Dewar (1959), Withler and Humphreys (1967), Withler and Morley (1968) and Stein (1975), who carried out a series of studies on the effect of low temperature on the storage of salmonid sperms.

When the cells are subjected to low temperatures, extracellular water crystallises first, causing an osmotic pressure on the cell membrane that eventually brings about the drainage of water from the cell into the extracellular medium. The success of the preservation of cells at low temperatures, thus depends on how well this dehydration of the intracellular medium can be brought about without causing any damage to the cell membrane. In order to achieve this, cryoprotectants are added into the extracellular medium. These cryoprotectants help in preventing the crystallisation of water within the cells even at very low temperatures and thereby indirectly protect the cell membrane. At the time of reuse, the samples are thawed to the room temperature. The rates of cooling and

thawing are highly critical and can cause damage to the cells if not properly controlled. The optimum rates of cooling and thawing differ from one cell type to another. Thus the success of the application of cryopreservation techniques largely depend on the type of cryoprotectant used, its quantity, the extender solution through which it is administered and the quality of the cells (sperms) employed. Besides an accurate knowledge of the mode of reproduction of the animal, the quality and percentage of motile sperms and their general characteristics, form an essential prerequisite.

The success achieved in the cryopreservation of semen in mammals prompted the earlier workers on fishes to adopt similar techniques, for fishes. However, it was soon realised that these techniques required suitable modifications when applied to fishes, as the structure, physiology and mode of fertilization in the teleost sperm were basically different from that of the mammalian sperm. As the teleost sperm lacks acrosome, the sperm penetration is affected through the micropyle on the egg membrane. Further the teleost sperm is also peculiar in the structure of the mid-piece. In fishes with external fertilization, (eg. M. cephalus) the sperm head is usually ovoid with a very small mid-piece, made up of a few mitochondria. Where as in the case of fishes with internal fertilization (eg. Poecilia reticulata) the head and mid-piece are

elongated, the latter containing a substantial number of mitochondria (Stoss and Donaldson, 1982). These differences in structure are considered to be responsible for the two different types of metabolic activities observed in the teleost sperms, namely, aerobic metabolism in the case of sperms of fishes with external fertilization (eg. salmonids) and anaerobic metabolism in the case of sperms of fishes with internal fertilization (eg. P. reticulata). Studies conducted by Mounib (1967) has clearly indicated that spermatozoa from oviparous fishes show limited glycolytic activity and depend mainly on oxidative metabolism for their energy requirements. The spermatozoa from viviparous fishes, however, convert exogenous sugars to lactic acid (anaerobic oxidation) and use this energy to prolong the duration of their motility just like the mamalian sperm (Gardiner, 1978). These differences in the metabolic activities explain why the salmonid sperm is viable for a longer period in an oxygen rich atmosphere unlike the mammalian sperm which exhibits longer viability in an inert atmosphere.

The major factors affecting the motility and viability of fish sperm are the temperature, salinity, pH and cation concentration. Dushkina (1975) observed that the sperms taken from the testes of dead Clupea pallasi and kept at 0.8°C were viable with nigh fertility rate for over two days. Studies on in vitro storage of salmonid sperm indicated that a reduction of temperatures to levels

just above freezing could prolong the viability of the spermatozoa (Withler and Morley, 1968; Hiroi, 1978; Stoss et al., 1978). Unlike in mammals, low temperatures were not found to cause any 'thermal shock' in the case of fish spermatozoa. However, fertility was found to reduce at a temperature of 0°C when a low density of spermatozoa was used, where as, at 5°C or by increasing the density of cells, better fertility was achieved (Billard and Gillet, 1975).

The effect of various salinities on the structure and fertilising ability of marine and freshwater fish spermatozoa were studied by Billard (1978). He opined that the medium in which the fertilization naturally occurred was not the optimum one for enhancing spermatozoan survival, the recommended optimum salinity of the medium being 20 ppt for the spermatozoa of marine fish and 7 ppt for that of fresh water fish. Earlier Billard and Jalabert (1974) showed that in trout, the eggs did not retain their fertility after remaining in freshwater for more than one minute. Similar results were also observed in the case of eggs of sea bream, sea bass and turbot, where the fertility of the eggs decreased within a few minutes of spawning. It was therefore clear that under natural conditions, the motility of the spermatozoa although short, was long enough for fertilization to occur, provided, spawning of both the partners was synchronous and there was sufficient amount of milt to fertilize the eggs. Since neither sea water nor freshwater

was found to be suitable for the survival of the spermatozoa, it became necessary to develop suitable media to be used in artificial fertilization of fish for prolonging the survivability of the gametes.

The studies on the influence of pH on the motility of sperms are limited and the available literature has been recently reviewed by Scott and Baynes (1980). Schlenk (1933) showed that the trout sperm could be activated in buffer at pH > 7.8 but not in the lower ranges. He claimed that seminal fluid had a pH of 7.3 and it was this factor that inhibited the sperm activity, in vivo. However, Schlenk and Kahmann (1938) later clearly demonstrated that the effect was greatly dependent on the presence of a suitable buffering system and that in the absence of such a system, sperm could be activated at a much lower pH.

The effect of ions on motility of spermatozoa has been reviewed by Scott and Baynes (1980), who report that weak solutions of KCl alone, result in permanent inactivation of spermatozoa due to osmotic shock, where as a combination of NaCl and KCl in the ratio of 140:20 millimoles could help in keeping the sperms inactive but alive, indefinitely. Earlier studies by Schlenk and Kahmann (1938) also showed that in the absence of Na^+ ions, even low concentrations of K^+ ions could inactivate spermatozoa where as in the presence of Na^+ ions, sperms could be activated even at higher concentrations of K^+ ions.

Baynes et al. (1981) demonstrated that in rainbow trout sperms, both Ca^{++} and Mg^{++} ions markedly prevent the inhibition due to low pH and K^{+} ions.

In teleosts the activation of spermatozoa is a 'once only' reaction. It is usually induced by the contact with the spawning medium (sea water in marine fish, freshwater in freshwater fish and ovarian fluid in viviparous fish). Within the gonad, the sperms remain alive but immotile. The exact mechanism of this process is not known. Once activated, the motility in the teleost sperm is confined to a short period of frenetic activity during which they should fertilize an egg. The short duration of activity is mainly because, the sperm is incapable of utilizing extracellular sources of energy (which they normally would not encounter in the medium). The quiescent nature of the spermatozoa in the seminal plasma, makes them suitable for short term storage. The important papers published on this aspect are by Scheuring (1925), Barret (1951), Blaxter (1955) and Ginsburg (1972).

The need for gaseous exchange for maintaining the viability of the sperms has been discussed in great detail by Stoss (1983). Air and Oxygen were found to be most suitable for maintaining cell viability, while the inert gases such as nitrogen, hydrogen, carbon dioxide or their mixtures were found to reduce storage capacity. Importance of adequate gas exchange in the storage of milt was

demonstrated by Truscott et al. (1968) in Salmo salar, by Withler and Morley (1968) in Oncorhynchus nerka and O. corbuscha and by Stoss et al. (1978) in S. gaidneri. Since oxygen enters the cells by diffusion, its availability to the sample depends on the surface area exposed to oxygen atmosphere. Increasing the sample depth decreases the storage ability of the cells. It is speculated that the variability reported in the case of the storage capability of salmonid sperms (from hours to weeks) might be due to inappropriate gaseous exchange. Though oxygenation of the milt sample prolongs the life of the spermatozoa, it may also lead to the evaporation of moisture from the sample. Buyukhatipoglu and Holtz (1978) have tried to overcome this problem by keeping Salmo gairdneri milt under moisture saturated with oxygen on air. Stoss et al. (1978) obtained encouraging results by storing semen(with antibiotics added) in open tubes placed in a sealed jar which is regularly flushed with air or oxygen.

As collection of milt under sterile conditions is difficult, the chances of bacterial and fungal infection of the stored milt is great. To prevent this antibiotics are made use of (Withler and Morley, 1968; Hiroi et al., 1973; and Hiroi, 1978). Stoss and Refstie (1983) recommended the use of 125 IU penicillin and 125 µg of streptomycin per ml of rainbow trout spermatozoa. The antibiotics are generally administered through a non-activating sperm diluent.

On the basis of the above information the important criteria to be taken into consideration while designing a short term storage of milt are

a) reduction in temperature

b) provision for gaseous exchange

c) prevention of bacterial growth

d) prevention of desiccation.

The preservation techniques of a short-term storage of spermatozoa are designed to reduce the metabolic activity of the cells in order to extend their life span (Stoss, 1983).

Short term storage of spermatozoa though possible, has many draw backs - need for oxygenation, prevention of contamination and desiccation. Harvey (1982) reported that even at -15°C cells are only partially dehydrated and bathe in a concentrated 'brine' of solutes that will damage the cell membrane. Addition of any amount of extracellular cryoprotectant was found to be unable to prevent the cell death within a few hours in the case of Brachydanio sperms. Preservation of spermatozoa for long period was possible only at temperatures below - 79°C (temperature of solid CO_2 at which dehydration will be complete).

As the principle involved in cryopreservation is one of gradual dehydration of cells without causing the damage of the cell membrane, various cryoprotectants are

used to prevent crystallisation of intracellular water. The cryoprotectants are generally added through the extender solutions. Initially extenders were formulated with a composition similar to that of seminal fluid with a high K^+:Na^+ ratio, without taking pH into consideration. From these simple inorganic extenders, the more complicated ones were developed later with organic compounds such as glucose, sucrose, fructose and egg yolk. The main function of the extender medium is to act as a vehicle for introducing the cryoprotectant into the extracellular medium. It should also bring about a dilution of the cells without activation, since the teleost sperm once activated cannot be cryopreserved and reactivated.

Cryoprotectants are of different types. The most commonly used cryoprotectants are glycerol and dimethyl sulphoxide (DMSO). Ethylene glycol and propylene glycol have also been used with limited success on the sperm of cod, salmonids and carp (Hoyle and Idler, 1968; Mounib et al., 1968; Kossmann, 1973 and Stein, 1979). Harvey (1982) has successfully used powdered milk as a cryoprotectant for Brachydanio. Though, the role played by cryoprotectants in improving the survival of spermatozoa is known, their exact mode of action is not clear. Rapatz and Luyet (1968) have suggested three categories of cryoprotectants namely (i) those with low molecular weight, eg. DMSO (ii) those with high molecular weight eg. lipoprotein from egg yolk and (iii) those with intermediate molecular weight such as glucose.

Lovelock and Bishop (1959) have suggested that non-electrolyte, low molecular substances such as DMSO act by reducing the ionic strength of the residual, unfrozen solution in and around the cell, during cooling. It is also reported that DMSO reduces the temperature at which intracellular freezing occurs from 0°C to -45°C and occasionally to -60°C (Leibo et al., 1978). This property of DMSO aids in the drainage of water from the cells at low temperatures. According to Mironescu (1977) DMSO stabilises membranes against damage by hypertonic solutions. It is probable that all these aspects collectively contribute toward improving the survival of cryopreserved material.

Investigations of Pace and Graham (1974) and Foulkes (1977) indicated that the low density lipoprotein fraction of egg yolk was an active constituent of cryoprotection of mammalian spermatozoa. Quinn et al. (1980) and Watson (1981) found that lipoprotein molecules could bind with plasma membrane. These properties of the low density lipoproteins were believed to give protection to cells during cryopreservation, although it was earlier believed that egg yolk, due to its high molecular weight and non penetrating nature, created a colloidal pressure in the external medium and that indirectly brought about dehydration. Jeyendran and Graham (1980) suggested that an interaction between egg yolk and DMSO with each other, together with their

combined action on the plasma membrane must be responsible for the cryoprotective effect, while Harvey (1982) opined that the osmotic pressure of the cryoprotectant was sufficient to inhibit motility. He found powdered milk added to the extender to be an excellent extracellular cryoprotectant. The rate of cooling and thawing are also very important in retaining the viability of the cells. At the optimum rate of cooling, ice formation first occurs extracellularly while the intracellular water supercools. The supercooling of the intracellular medium is enhanced by the presence of the cryoprotectant. However, the equilibration time required by the cryoprotectant to interact with the membrane is significant as rapid cooling would facilitate the formation of ice crystals which may damage the cell membrane. If the rate of cooling is too slow, the hyperosmotic state of the medium (high external salt concentration) would cause irreparable membrane damage.

The physiological processes occuring during thawing are not clearly understood. It is presumed that during thawing, intracellular ice melts before the extracellular and this causes a further transport of water out of the cell. This increases stress on the membrane which is already sensitised by freezing (Baynes, 1984). Rapid thawing was found to be less damaging (Shimada and Asahina, 1975). Though dilution with extender solutions

prevent motility, motility of the sperms is spontaneously induced on thawing the samples. The duration of motility in the post thawed samples is found to be relatively less than in the fresh samples.

In the background of the above knowledge, two experiments on the cryopreservation of milt of M. cephalus and L. parsia were planned and carried out after studying the general spermatological characteristics.

MATERIALS AND METHODS

Spermatology

(i) Sperm Count: Fresh milt was collected from live specimens of both M. cephalus and L. parsia using a canula into clean dry 5 ml. glass vials. Since the milt of both the species were highly viscous, they had to be suitably diluted with marine fish ringer solution before making the counts. By trial and error method it was found that a dilution ratio of 1:800 and 1:2000 (milt: marine fish ringer) was suitable for M. cephalus and L. parsia respectively. 40 ml of marine fish Ringer solution was taken into a clean dry beaker and 0.05 ml of milt from M. cephalus was transferred into it using a

micropipette. The pipette was repeatedly rinsed with the solution in the beaker inorder to wash down the milt adhering to the sides of the pipette. In the case of L. parsia 0.05 ml of milt was diluted with 100 ml of marine fish Ringer solution by the same procedure. The diluted milt was shaken well inorder to ensure a thorough mixing of the milt and the diluent. A few minutes later, when the cells became inactivated, a drop of the diluted milt was placed on the counting chamber of the Neubauer hemocytometer and a coverslip was carefully placed over it. The excess of solution was blotted out using a tissue paper and the hemocytometer was observed under an Olympus Monocular compound microscope at 10 x 40 x magnification. The volume of the solution trapped in the counting chamber under the coverslip was 0.001 ml. The number of cells, distributed in about 80 small squares were counted by the standard method for counting red blood corpuscles. Each sample was counted in four replicates. The milt samples from 10 fishes were taken for determining the mean value. The number of cells per ml (x) was estimated using the formula:

$$x = \frac{\text{No. of cells counted x 4000x dilution factor}}{\text{No. of squares counted}} \times 1000$$

(ii) Sperm Motility: Spermatozoan motility was assessed according to the criteria established by Emmens (1947) and Jaspers (1972), with slight modification.

A drop of milt taken from a live ripe (Vth stage) specimen of *M. cephalus* or *L. parsia* was placed on a clean dry glass slide with a drop of marine fish Ringer solution. A coverslip was carefully placed over it and the material was immediately observed under a compound microscope (Olympus) at a magnification of 10 x 10x and 10 x 40x. Three main types of movements could be observed -

1. rapid progressive or shooting movement
2. sluggish or lethargic movement
3. vibration in loco.

Each sample was observed in at least three fields of view and a quick eye estimation of the approximate percentage of spermatozoa belonging to each of the above categories was done. On the basis of this a motility score was given to each sample, as per the table below. The method was standardised by repeating the process with a number of samples.

Criteria	Motility Score
1. Ninety percent or above of the sperms exhibiting rapid progressive or shooting movement	5
2. Seventy five percent or more exhibiting rapid progressive movement, ten percent sluggish and the rest immotile	4
3. Fifty percent exhibiting rapid progressive movement, twenty five percent sluggish and ten percent vibrating in loco and the rest immotile	3

Criteria	Motility Score
4. Twenty five percent exhibiting shooting movement, fifty percent moving sluggishly, ten percent vibrating in loco and the rest immotile	2
5. Occasional sperm shooting, ten percent showing sluggish movement, fifty percent vibrating in loco and the rest immotile	1
6. No shooting movement. Occasional sperm moving sluggishly. About ten percent vibrating in loco. Majority immotile	0.5
7. Completely immotile	0.0

Only samples with a motility score of 4 and 5 were chosen for cryopreservation. In L. parsia the milt collected from 2 or 3 specimens of more or less same size and weight were pooled together.

Cryopreservation

The experiments on cryopreservation were conducted with two cryocans procured from the Kerala Livestock Development and Milk marketing Board, Muvattupuzha (plate XXXVII, Figs 1 and 2).

The milt from fifth stage males of both the species ranging in size from 360 mm to 405 mm (total length) in M. cephalus and 110 mm to 175 mm in L. parsia was collected using a canula (plate XXXVII, Fig. 3) and transferred immediately into a 5 ml glass vial where it was diluted with one of the following extender solutions.

PLATE XXXVII

Fig. 1. Photograph of the cryocan used for cryo-preserving the frozen samples of milt (as viewed from above). Arrows indicate the labelled canisters.

Fig. 2. Apparatus used for cryopreservation of milt.
1= Cryocan for storing liquid nitrogen.
2= Cryocan for cryopreserving milt.
3= Thermocol container for freezing samples.
Arrow indicates the metal frame used for keeping the vials and straws while freezing.

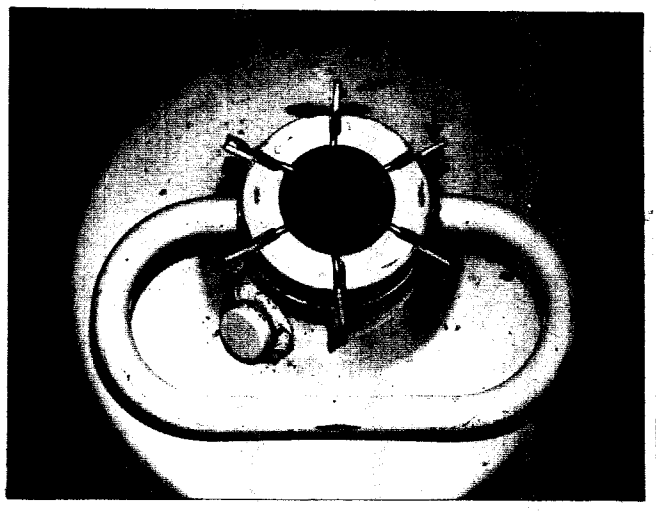
Fig. 3. Collection of milt from oozing specimen of Liza parsia, using canula. CN=Canula; MT= Milt.

Fig. 4. Sealing of the extended milt in the glass vial with insulation tape. MT=Milt; TP= Insulation tape; VI Vial.

Fig. 5. Thermocol container with the metal frame, used for freezing milt samples. LD= Lid; TC=Thermocol container; Arrow indicates metal frame.

Fig. 6. Transfer of frozen vials into canisters with liquid nitrogen.
1= Lid of the cryocan;
2= Freezing chamber;
3= Cryocan;
4= Precooled forceps;
5= Canister with liquid nitrogen; Arrow indicates frozen vial.

PLATE XXXVII



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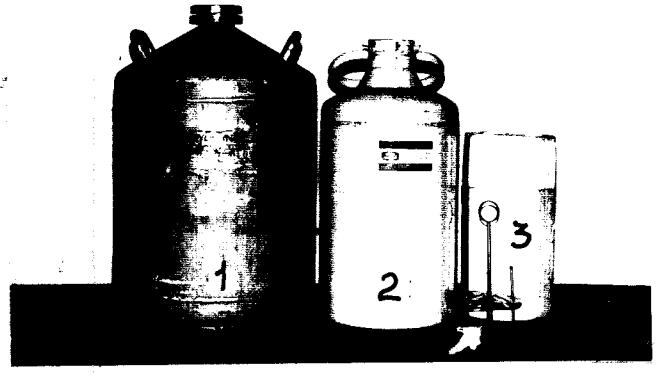

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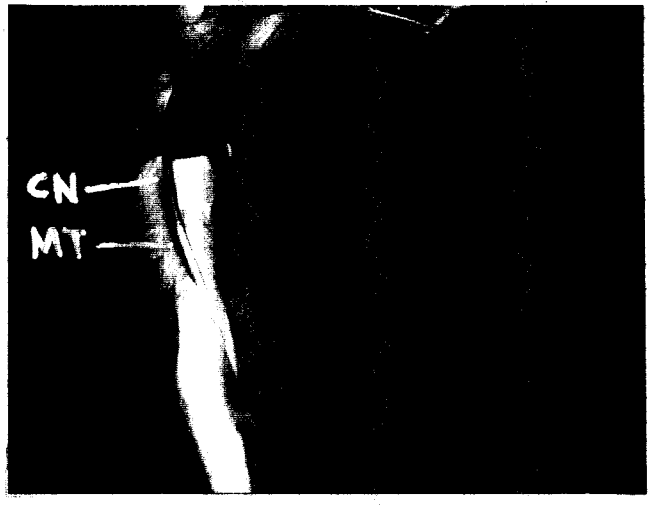

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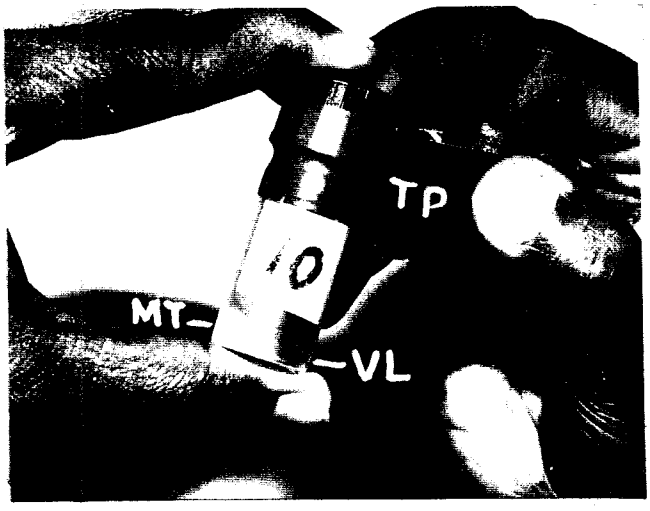

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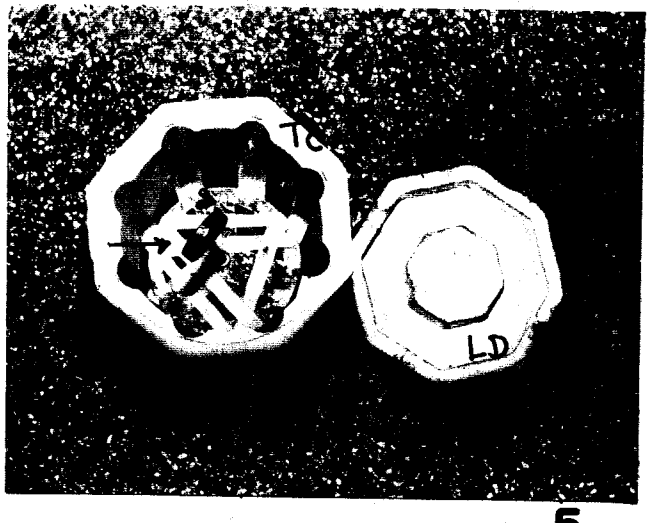

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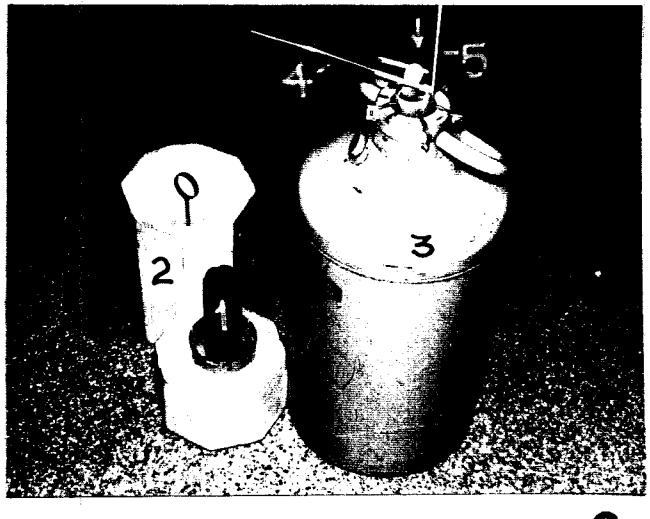

6

Extenders	Composition
A	Marine fish Ringer solution
B	5% glycerine in Marine fish Ringer
C	10% glycerine in Marine fish Ringer
D	15% glycerine in Marine fish Ringer
E	5% Dimethyl sulphoxide (DMSO) in Marine fish Ringer
F	10% DMSO in Marine fish Ringer
G	15% DMSO in marine fish Ringer
H	Chao's extender: 80 ml of 5% glucose in marine fish Ringer and 20 ml of DMSO
I	Tris egg yolk citrate: 67.2 ml Tris buffer 20 ml egg yolk 6.4 ml double distilled water 0.03 gram streptomysin
J	Mounib's extender: 4.28 g sucrose 1.00 g $KHCO_3$ 0.2 g Reduced glutathione 100 ml dist. H_2O One part of DMSO is added to 7 parts of the above solution just before use.

The exact ratios of dilution are given in the tables 24-26. Antibiotic streptomysin was used only in one extender solution (I). The diluted milt was drawn into labelled plastic straws (French straws) of 0.5 ml and 0.25 ml capacity. The ends of the straws were then sealed using a polyvenyl sealing medium after leaving an air gap of 2 cms. About 0.2 ml of extended milt was also taken in flat bottomed labelled screw capped corning

vials of 5 ml. capacity, and sealed with insulation tape (plate XXXVII, Fig. 4). The straws and vials containing the extended milt were exposed to liquid nitrogen vapours by placing them on a specially designed metal frame (plate XXXVII, Fig. 5) kept inside a thermacol container containing a layer of liquid nitrogen at the bottom. Care was taken to ensure that the liquid nitrogen did not directly touch the straws or the vials. After five minutes of exposure to the liquid nitrogen vapours, the vials and straws containing the frozen samples were transferred to labelled canisters containing liquid nitrogen, using precooled forceps (plate XXXVII, Fig. 6). The canisters were then placed in the cryocan. The motility score was estimated in the thawed samples at five day intervals.

Thawing:

The cryopreserved samples were thawed at a temperature between 26-30°C in water baths. After the ice in the samples had dissipated, the vials and straws were removed from the water bath and the reactivated sperms were tested for motility and fertility.

Fertilization Experiments

Fertilization experiments were conducted only in the case of L. parsia since female spawners of M. cephalus

were not available. In. L. parsia, two fertilisation experiments were conducted, one with the milt cryopreserved for 15 days and the other with the milt cryopreserved for 20 days. The female spawners for both the experiments were procured from the Cochin barmouth region and transported live to the Prawn Culture Laboratory of Central Marine Fisheries Research Institute, at Narakkal, where the experiments were conducted.

The female spawners 16.9 to 22.5 cms in length and weighing about 55 to 110 grams were artificially induced to spawn by administering pituitary injections as per the method standardised by the Mullet culture section of the CMFRI at its Narakkal centre. The eggs were stripped into a clean dry tray from which about 300 eggs were carefully transferred into labelled cavity blocks using a spatula. Two drops of thawed milt were added to the eggs followed by two ml of filtered sea water (31 ppt). The contents of each cavity block was mildly agitated by bubbling air using a micropipette to ensure proper mixing of gametes. Twenty minutes later, they were observed under the microscope for cleavage. The milt cryopreserved in extenders from A to I were used in the first experiment while those from A to J were used in the second experiment. Samples preserved in both vials and straws were used in the first experiment while only those kept in vials were used in the second experiment. With each experiment, a

control experiment was simultaneously conducted. For the control, milt freshly stripped from live males was used to fertilize eggs from the same female spawner.

RESULTS

1. Sperm count:

The estimated number of sperms per ml from ten fishes, in the size range of 36.5 to 40.5 cms and weighing 495 to 655 grams in M. cephalus and 11 to 17.5 cms and 20 to 50 grams in L. parsia are given in the tables (Table 21 and 22). The mean overall sperm count in M. cephalus was 7.62×10^{10}/ml of milt and that of L. parsia was 2.69×10^{10}/ml of milt.

2. Motility score:

The motility score of fresh milt of L. parsia and M. cephalus diluted with sea water at room temperature is given in the table. It is observed that within 15 minutes of dilution the motility score of both the samples is reduced to '2' (Table 23).

At 5°C, the undiluted milt of both the species could be maintained in flat bottomed glass vials upto 48 hours without great difference in the motility. When this milt was diluted with sea water the motility score

TABLE 21. Sperm count of *M. cephalus* milt

S. No.	Total length of fish (mm)	Weight of fish in grams	No. of /ml of sperms/ milt
1.	405	650	7.93×10^{10}
2.	365	495	7.16×10^{10}
3.	385	510	7.98×10^{10}
4.	395	655	8.20×10^{10}
5.	414	520	7.72×10^{10}
6.	395	630	7.92×10^{10}
7.	390	510	7.81×10^{10}
8.	360	495	6.66×10^{10}
9.	384	550	7.54×10^{10}
10.	381	625	7.28×10^{10}
Mean	387.3	564	7.622×10^{10}
S.D	$\pm$15.6974	$\pm$64.218	$\pm 0.46145 \times 10^{10}$

TABLE 22. Sperm count of *L. parsia* milt

S. No.	Total length of fish (mm)	Weight of fish in grams	No. of /ml of sperms / milt
1.	132	22.0	2.3×10^{10}
2.	155	40.0	2.75×10^{10}
3.	175	50.0	3.15×10^{10}
4.	165	42.3	2.65×10^{10}
5.	165	45.0	3.40×10^{10}
6.	132	20.0	1.90×10^{10}
7.	155	35.0	2.75×10^{10}
8.	145	30.0	2.60×10^{10}
9.	142	25.0	2.40×10^{10}
10.	159	40.0	3.00×10^{10}
Mean	152.300	34.900	2.69×10^{10}
S.D.	$\pm$13.689	$\pm$9.7103	$\pm 0.433205 \times 10^{10}$

TABLE 23. Motility score of fresh milt of *L. parsia* and *M. cephalus* diluted with sea water at room temperature.

Time Mins.	*L. parsia*	*M. cephalus*
0	5	5
5	3	4
10	3	3
15	3	3
20	2	2
30	1	0

rapidly reduced to '0' within 15 minutes.

Milt extended with egg yolk citrate containing streptomycin was maintained at 5°C for 7 hours, without change in the motility score. On dilution with sea water there was high motility which was gradually reduced to 0.5 within 15 minutes.

The motility score of the cryopreserved samples taken at 5 day intervals, are given in the tables (24-26). In the case of M. cephalus milt, it is seen that except for the extender C, all other extenders have failed to retain any motility score in the straw preserved samples, even for five days. In the vial preserved samples, extenders C and E gave the best results with a motility score of 4 even after 15 days of cryopreservation. Samples extended with B gave a motility score of 2; those extended with F, H and I showed a motility score of 1 and with A and G, a motility score of 0.5 after fifteen days of cryopreservation (Table 24).

In the case of L. parsia, two sets of cryopreservation experiments of 15 and 20 days duration were conducted. In this case also, the straw preserved samples (except E in first experiment) did not retain any motility after 15 days of cryopreservation.

In the first set, maximum motility was observed in samples extended with B and cryopreserved for a

TABLE 24. Motility score of cryopreserved *M. cephalus* milt.

Extender used	Dilution Milt:Extender	Straw(s)/ Vial(v)	Motility after 5 days	Motility after 10 days	Motility after 15 days
A	1:1	V	2.0	1.0	0.5
	1:1	S	0.0	0.0	0.0
B	1:1	V	3.0	2.0	2.0
	1:1	S	0.0	0.0	0.0
C	1:1	V	4.0	4.0	4.0
	1:1	S	1.0	0.0	0.0
D	1:1	V	0.0	0.0	0.0
	1:1	S	0.0	0.0	0.0
E	1:1	V	5.0	4.0	4.0
	1:1	S	0.0	0.0	0.0
F	1:2	V	2.0	2.0	1.0
	1:2	S	0.0	0.0	0.0
G	1:8	V	0.5	0.5	0.0
	1:8	S	0.0	0.0	0.0
H	1:3	V	3.0	2.0	1.0
	1:3	S	0.0	0.0	0.0
I	1:1	V	3.0	2.0	1.0
	1:1	S	0.0	0.0	0.0

maximum period of 15 days in vials. While samples extended with A and H cryopreserved for the same period showed no motility, sample C showed a motility score of 0.5; E and I a motility score of 1; D and F, 2 and G, 3 (Table 25).

In the second set, only vials were used. The highest motility score was observed only in 4 samples after a duration of twenty days. These were the samples extended with C, D, F and G. In the second set, samples extended with A, B and I showed no motility, while samples extended with E, H and J showed a motility score of 1,2 and 0.5 respectively after a period of twenty days (Table 26).

3. Fertilization:

To test the viability of the cryopreserved milt samples of L. parsia, two experiments on fertilization were conducted, with eggs obtained from females of the species that were induced to spawn in the laboratory (NPCL).

Experiment - I: It was conducted on 26th of June 1984. Milt samples cryopreserved for 15 days were tested. Fertilization (80%) was obtained only in the case of one sample which was extended with the extender solution B and cryopreserved in the vial. It had a motility score of 4. All other samples failed to fertilize the eggs.

TABLE 25. Motility score of cryopreserved milt of *L. parsia*

Experiment - 1.

Extender used	Dilution Milt:Extender	Straw(S)/ Vial(V)	Motility after 5 days	Motility after 10 days	Motility after 15 days
A	1:1	V	0.0	0.0	0.0
	1:1	S	1.0	0.5	0.0
B	1:1	V	4.0	4.0	4.0
	1:1	S	0.0	0.0	0.0
C	1:2	V	3.0	1.0	0.5
	1:2	S	0.0	0.0	0.0
D	1:1	V	4.0	2.0	2.0
	1:1	S	0.5	0.0	0.0
E	1:2	V	4.0	3.0	1.0
	1:2	S	2.0	1.0	0.5
F	1:2	V	3.0	3.0	2.0
	1:2	S	1.0	0.5	0.0
G	1:1	V	4.0	3.0	3.0
	1:1	S	0.0	0.0	0.0
H	1:1	V	2.0	0.5	0.0
	1:1	S	1.0	0.0	0.0
I	1:2	V	3.0	1.0	1.0
	1:2	S	0.0	0.0	0.0

The control experiment with milt stripped freshly from the males, also showed 80% of fertilization.

Most of the straw preserved samples had coagulated. The sealing medium had come off from the sealed end in most cases, allowing the entry of liquid nitrogen into the sample contained in the straw. Even the uncoagulated straws did not show any motility score greater than 0.5.

In the fertilized eggs (both control and experimental) the first cleavage was observed twenty minutes after the addition of the milt to the eggs. Subsequent cleavages were followed upto the 16 cell stage. About 30% of the fertilized eggs hatched into normal larvae in both the control as well as the experimental cases.

Experiment - II: The second experiment was conducted on the 15th of August 1984, with samples of milt, cryo-preserved for 20 days. The eggs for this experiment were obtained from two female spawners of *L. parsia* induced to spawn in the laboratory. Only vial preserved samples were used for this experiment.

The first/spawned at 2.40 a.m. The eggs were/fish found to be of poor quality since many of them did not float. The rate of fertilization was very low and only about 30% fertilization could be observed in the control. Samples extended with A, B, C, D and J were tested. Only

TABLE 26. Motility score of cryopreserved milt of *L. parsia*

Experiment - II

Extender	Dilution Milt: Extender	Motility after 5 days	Motility after 10 days	Motility after 15 days	Motility after 20 days
A	1:1	4.0	2.0	0.0	0.0
	1:3	4.0	2.0	0.0	0.0
B	1:2	2.0	2.0	1.0	0.0
C	1:3	4.0	4.0	4.0	4.0
D	1:3	5.0	4.0	4.0	4.0
E	1:3	4.0	3.0	1.0	1.0
F	1:4	5.0	4.0	4.0	4.0
G	1:2	5.0	4.0	4.0	4.0
H	1:2	4.0	3.0	3.0	2.0
I	1:2	0.0	0.0	0.0	0.0
J	1:3	2.0	1.0	1.0	0.5

samples extended with C and D showed a motility score of 5 but fertilized only about 30% of the eggs. The first cleavage of the eggs occurred 25 minutes after mixing the milt and eggs.

The second fish spawned at 5.05 a.m, liberating good quality eggs. Samples extended with E, F, G, H and I were tested. The milt samples extended with F and G with a motility score of 5, gave above 70% fertilization. In the samples extended with E and H with a motility score of 2, only 5% fertilisation was obtained. The control experiment conducted with freshly stripped milt also indicated fertility above 70%.

DISCUSSION

As mentioned earlier, the investigations on cryopreservation of gametes in mullets are very limited. The only species studied so far is M. cephalus. Hwang et al. (1972) investigated the effect of a series of temperatures ranging from 24°C upto -20°C on M. cephalus sperms, extended with marine fish ringer solution using glycerine and DMSO as cryoprotectants.

In 1973, Chao et al., attempted both low temperature and cryogenic preservation of M. cephalus milt extended with nutrient diluents consisting of various concentrations of sodium citrate, glucose and lactose. Of these, 2% sodium citrate, 5, 10 and 12% glucose and 24% lactose were found to be suitable for short term preservation of samples at 5°C. Further Chao et al. (1975) studied the spermatological characteristics of M. cephalus milt and the toxic levels of glycerine and DMSO. The best results reported by them (after a period of one year and four days) were in the case of samples stored in 10% glycerine and 10% DMSO. The former had a fertilization rate of 2.4% with a hatching rate of 52.45%, while the latter had a fertilization rate of 2.7% and a hatching rate of 31%. The results of these studies indicated that, long term preservation of mullet sperm

The results of the present experiments showed that marine fish Ringer alone without any cryprotectants was not sufficient for long term preservation. Glycerine and DMSO administered through marine fish Ringer were found to be effective at 5%, 10% and 15% levels in both the experiments. However, 5% DMSO did not yield good results. Tris-egg yolk citrate, that was successfully used for cryopreservation of bull semen was not found to be useful in the present study. Similarly, glucose based Chao's extender and sucrose based Mounib's extender did not yield good results. The French Straw technique was a complete failure in the present study. It was therefore not made use of in the second experiment. The main reason for this failure appears to be improper sealing since in many of the samples liquid nitrogen seemed to have entered the straw and the contents of the straw had coagulated. No coagulation was observed in the samples stored in vials. One of the reasons for the success of the vials may be the presence of a large air gap immediately above the layer of extended milt. In the case of the straws, the air gap was in contact with only a very small area of extended milt which might have prevented proper gaseous exchange during the period of equilibration and freezing. Since the cryocans used for the study were obtained on a loan basis from Kerala Live-stock Development and Milk Marketing board for three months only, both the experiments conducted were of short

duration. The variability of results in the two experiments indicate that further experiments have to be conducted before evolving a perfect technique.

The high percentage of fertilization obtained in the case of L. parsia is encouraging and shows that this technique is a suitable one for this particular species of mullets.

Bhowmick and Bageni (1971) attempted storage of milt from common carp at temperature ranges from 0 to 5°C, with extenders such as sodium citrate, phosphate buffer, Holtfreter's solution and frog Ringer solution. In 1983 under a collaborative programme between the University of Delhi and the Central Inland Fisheries Research Institute, Cryopreservation studies on the milt of Labeo rohita and silver carp were attempted. A motility and fertility rate of 70% was observed after a maximum period of 21 days of cryopreservation (Kuldip kumar - personal communication). Apart from these studies on fresh water fishes, no work on cryopreservation of teleost milt have been reported from India. Under these circumstances, the present work might be of value in formulating a suitable extender medium and designing an appropriate method for the cryopreservation of cultivable species of marine fishes.

During the present studies it was noticed that while oozing males of M. cephalus could be obtained in

large numbers from the wild, the females obtained at the same time, were only in the third stage of maturity. Though this prevented the worker from conducting fertility tests of cryopreserved samples, it also showed how important, cryopreservation was in ensuring the availability of the sperms at the appropriate time.

Though vials were found to be successful in the present study, the straw technique is more widely used and is recommended by many workers since it is more compact, inexpensive and easily disposable. It is, therefore, suggested that more work be done in this area. The cooling and thawing time used for both the straws and the vials were the same in the present experiment and it was found that while the samples in the vials gave good results, the straws failed. It is, therefore, speculated that these timings might have some effect on the fertility of the sample. The ratio of the extender:cryoprotectant and extender: sample have to be standardised for each extender and each species.

The techniques involved in long term preservation of sperms is quite complicated and requires appropriate standardisation of each of the steps involved. The results of the preliminary experiments and observations presented here indicate that with further refinement of techniques, it would be possible to develop a viable method for long term cryopreservation of the milt of Indian mullets.

S U M M A R Y

1. The thesis presents a comprehensive account of the spermatogenesis in Mugil cephalus Linnaeus and Liza parsia (Hamilton - Buchanan), together with the results of preliminary investigations on the cryopreservation of their milt.

2. Following a review of literature on the biology and reproductive physiology of these species and material and methods of investigation, the study begins with a detailed description of the structure and organisation of the male reproductive system in the two species.

3. The male reproductive system consists of a pair of elongated testes, with a vas deferens running all along the length of each testis. Posteriorly the two vasa defferentia unite to form a common duct that opens out at the genital pore. Initially the testes make their appearance as two thread-like strands of tissue attached to the body wall at the dorsal side by means of the mesorchium. But as development proceeds, the testes grow in

size, become flattened and reorient themselves in such a way that the mesorchium in the mature gonad, appears to be connected from the inner lateral side of the testis to the dorsal body wall.

Internal testicular structure reveals that the testes of M. cephalus and L. parsia belong to the 'lobular type' since the body of the testis is made up of a number of seminiferous lobules of variable diameter. The lumen of the seminiferous lobules are continuous with the vasa efferentia that lead to the vas deferens in each testis. The testis is further described as the 'unrestricted type' since each seminiferous lobule is made up of seminiferous cysts containing germ cells at various stages of spermatogenesis, all along its length. Each cyst is bordered by cytoplasmic extensions from the Sertoli cells present along the inner wall of the seminiferous lobule. Externally the lobules are lined by spindle shaped boundary cells. The interlobular space is made up of connective tissue with a few Leydig cells and blood vessels.

The testes during the process of maturation, undergo changes in their morphological and histological characters. On the basis of these characteristics,

six stages of maturity are identified; these are: immature, maturing I, maturing II, mature, oozing and spent. The characteristic features of each stage are discussed in detail.

6. The environmental parameters such as temperature, photoperiod, salinity and dissolved oxygen, monitored throughout the period of study, are correlated with the condition factor and the gonado-somatic index of the two species. The study reveals that under natural conditions of the study area, where these parameters are found to fluctuate within a small range, the reproductive cycle of the two species are governed by an endogenous rhythm. M. cephalus shows distinct peaks of GSI during the months of November, May and June, while L. parsia has a single protracted period of high GSI extending from October to May.

7. The ultrastructure or cellular organisation at various stages of spermatogenesis in the two species is studied by electron microscopy. The primordial germ cell is characterised by an irregular outline, eccentric nucleus with distinct nucleoli, large number of cytoplasmic organelle and low nuclear/cytoplasmic ratio. The spermatogonia are the largest cells in the testes, each

with a regular membrane, an electron-dense cytoplasm and a large nucleus with granular chromatin and a smooth outline. The primary and secondary spermatocytes are synchronously dividing cells which show a conspicuous decrease in cell size and an increase in the nuclear/cytoplasmic ratio. The spermatids are characterised by a series of sub-stages, showing gradual condensation of chromatin material, formation of flagellum mid-piece and reorientation of the head with respect to the tail. The sequence of spermatogenesis and the structure of the various cell types are found to be almost similar in the two species. However, differences are observed in the actual size of the cells and the nuclear/cytoplasmic ratios. The important structural features of the spermatozoa of the two species are compared with that of L. auratus and L. dumerili.

8. An estimation of protein, carbohydrate, lipid and cholesterol in the testis, muscle, liver and blood serum, during the different maturity stages reveals that there is a distinct depletion of these body resources from the somatic tissue during maturation. This depletion is found to be partly due to the translocation of these substances to the testes for the synthetic activity during

gametogenesis and partly for meeting the energy requirements of the fish during the final stages of maturation, when the fish abstains from feeding.

9. The distribution of the different types of proteins, carbohydrates and lipids in the various cell types of the testicular tissue at each maturity stage is studied by the qualitative histochemical tests. The testicular tissue is positive to proteins with acidic, basic, -SH, -SS, tyrosyl and tryptophanyl groups. But the intensity of reaction for each group is seen to be different in each cell type. An increase in the intensity of staining of the basic proteins is seen in the final stages of spermatogenesis. The tests for carbohydrates reveal the presence of traces of polysaccharides, glycogen and sulphated acid-mucopolysaccharides, in the testicular connective tissue as well as the various cell types. Histochemical tests for lipids show that the testicular lipids are mostly neutral lipids, phospholipids and cholesterol and are found predominantly in the connective tissue and the interstitial cells.

The spermatological studies on the milt of M. cephalus and L. persia reveal that M. cephalus has a mean sperm count of 7.62×10^{10}/ml of milt, while it is

about 2.69 x 10^{10}/ml of milt in L. parsia. The motility scores of the milt samples are estimated by using a six point scale. It is observed that within 15 minutes of dilution of milt with sea water at room temperature, the motility score of the milt samples of both the species, reduced to '2'. The undiluted milt of both the species, however could be preserved at 5°C up to 48 hours without reduction in motility.

11. Experiments on cryopreservation of milt of both the species are carried out using ten extender solutions. Marine fish Ringer solution formed the major component of seven of these extenders, with 5%, 10%, and 15% glycerine and dimethyl sulphoxide (DMSO) acting as cryoprotectants. The other three extenders used are, Chao's extender, tris egg yolk citrate and Mounib's extender. The motility score of the milt cryopreserved in these extenders are determined at five days interval and the data are presented and discussed.

12. Two fertilization experiments are conducted with the milt of L. parsia cryopreserved for 15 and 20 days and the eggs induced to spawn in the laboratory by administering pituitary injections. A fertilization rate of about 70 to 80% was obtained in both cases.

The results show that marine fish minger solution with 5%, 10% and 15% glycerine or 10% DMSO are more effective in cryopreservation of the milt than the other extenders used in the present study.

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